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THE UNIVERSITY OF ALBERTA

METABOLITES OF MYCENA CITRICOLOR AND THE SYNTHESIS OF CERATENOLONE

by

CLAUDE DUFRESNE

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

SPRING 1987

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Supervisor

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External Examiner

Date October 21, 1986

A mes parents

Abstract

Chemical studies relevant to two plant diseases, the American Leaf Spot disease of coffee and the Blue Stain disease of lodgepole pines, have been carried out.

Citricolic acid, a metabolite of the fungus *Mycena citricolor*, causal agent of the coffee disease, has been shown to possess structure 1. The structure was elucidated by a combination of physical and chemical methods. Citricolic acid (1) is a new type of natural product which appears to be a degraded steroid. A possible biogenesis of 1 is discussed. The role of oxalic acid, produced by *M. citricolor*, in causing the symptoms of the disease is also discussed.

A synthesis of ceratenolone (2), a metabolite of *C. minor*, one of the fungi involved in the Blue Stain disease, is presented. The formation constants for the ceratenolone / iron (III) tris complex were found to be $K_1 = 10^8$, $K_2 = 4 \times 10^7$, and $K_3 = 1.5 \times 10^6$, $\log\beta_3 = 21.8$. The molar absorbtivity of the mono, bis, and tris complexes were found to be $\epsilon_1 = 1000$, $\epsilon_2 = 1550$, and $\epsilon_3 = 15700$.

(1: 6(S),10(S)-Dihydroxy-10(S)-(5-hydroxy-1,4,5 trimethyl-2(E)-hexene)-9(S)-methyl-5-oxa-4-oxo-tricyclo $|7.3.0.0^{2,6}|$-2-dodecene)

(2: 2-ethyl-2-methyl-4-hydroxymethylene-5-(1-methylpropyl)-5-cyclohexene-1,3-dione)

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I. INTRODUCTION

This thesis describes a part of the efforts made in these laboratories towards the understanding of the chemical basis of the interaction of phytopathogenic fungi with plants.

One of the most serious diseases of coffee in Latin America is that caused by the fungus *Mycena citricolor* (Berkeley et Curtis) Saccardo, commonly known as the American leaf spot disease of coffee or "ojo de gallo" (eye of the rooster). In Costa Rica, as an example, this disease has been estimated to cause an annual loss of up to 50% of the coffee crop.[1] In spite of its importance, the disease has not been studied extensively.

The American leaf spot was first observed on coffee in Columbia in 1876.[2] The fungus was later determined to be a Basidiomycete and named *Omphalia flavida*. In 1950, Dennis[3] decided that the correct designation for the fungus should be *Mycena citricolor* and this is the binomial generally accepted at the present time.

M. citricolor exerts its major pathological effect on coffee and other host plants by bringing about severe defoliation. The fungal infection causes light brown circular or ovoid spots on the leaves, 0.5 to 1.0 cm in diameter, with little or no chlorosis in the surrounding tissue. When the necrotic lesions are formed along the principal vein on the leaf, the leaf drops, giving rise to premature defoliation.[4] Once the disease has been

established in a crop, defoliation occurs within a relatively short period (about 8 days).[5] Reduced coffee production is thought to be due to utilization of food reserves by the plant to produce leaves instead of fruit. The few fruits produced may also be attacked by the fungus and fall.

If the leaves bearing the fungus lesions remain under conditions of constant high humidity, the fruiting bodies (gemmae) appear as small yellow structures usually at the edge of the lesions. Relatively small numbers of gemmae, generally 1-20 at any period, are formed on each necrotic spot. The gemmae are the only known form of innoculum, being spread from tree to tree by wind and splashing rain drops. The gemmae also stick to insects, birds, and workers and are thereby dispersed over long distances.

Altogether *M. citricolor* attacks about 150 species belonging to more than 45 families. The fungus, in addition to being a severe pathogen of coffee, also causes a serious infection on cinchona, cacao, and citrus. Coffee is extremely susceptible to this fungus[6] and it is usually grown under conditions (high temperature and humidity, shade) which favor the development of this forest pathogen.[1] Varieties of coffee resistant to this disease are not known, perhaps due to the non-specialized parasitism of the fungus.[1]

The severe defoliation brought about by *M. citricolor* can not be explained by simple mechanical injury, since

leaves with large perforations at the base of the laminae remain attached to the plant for considerable periods. Thus auxins are implicated in the development of the disease.[11] Further studies have shown that the application of indoleacetic acid delays leaf drop considerably. This result suggests that the fungus interferes with the normal flow of auxin from the blade to the petiole. Subsequently it has been found that *M. citricolor* produces an oxidative enzyme in culture which causes rapid inactivation of indoleacetic acid. It is thought that this enzyme may be responsible for the premature abscission of infected leaves.[12] The effects of the production of indoleacetic acid oxidase *in vivo* on abscission are debatable,[13] and the role of other possible inducers of abscission deserves investigation.

Control methods for the American leaf spot disease rest primarily with the use of fungicides. Copper fungicides have been used with promising results, but the heavy rainfall in the Costa Rican coffee zone makes it difficult to maintain an adequate film on the foliage, and frequent spray applications are economically unfeasible. Certain mercury-containing fungicides control this coffee disease,[7] but unfortunately field tests with mercury fungicides have shown the presence of a toxic mercury residue in the harvested coffee beans, making these fungicides unsuitable. Lead arsenate, another eradicant fungicide, has been used to fight the disease with very

good results. Recently, however, the use of lead arsenate has been regulated due to the danger of toxic residues in the coffee beans. The regulation prohibits the use of lead arsenate during the maturation and harvesting seasons, which, because of the heavy rains, is precisely the time when *M. citricolor* attacks. In addition, there has been a movement within the chemical industry to stop the production of lead arsenate because of its toxicity, restricted use, and environmental pollution properties.[8] Other fungicides have been used but are less effective than lead arsenate and lower the quality of the coffee beverage.

M. citricolor is a luminescent fungus and this property has been used as an indicator of disease incidence on host plants.[6] The preparation and purification of the substance responsible for luminescence has been reported.[9] To date no structure for the active substance has been proposed. However, the structure of the fluorescent constituents of a related bioluminescent fungus, *Lampteromyces japonicus (Clitocybe illudens)*, have appeared.[10] The authors point out that one compound responsible for light emission of these fungal extracts has u.v. peaks identical with those reported for fungal luciferin from *M. citricolor*.

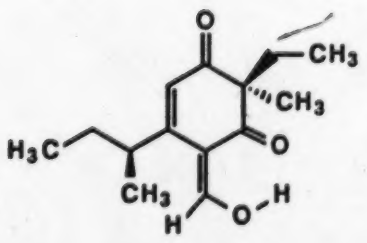
Since disease is generally the result of an interaction between host and pathogen, any contribution toward a better understanding of the parasitism has to be based on separate studies on the pathogen and host as well

as on the host-parasite complex. As part of a general program, funded by the International Development Research Centre of Canada, to develop strategies for the control of this disease, we undertook a study of the biology and chemistry of the disease in co-operation with research scientists in the fields of plant science and agronomy. The work described herein is part of a chemical study of the metabolites produced by *M. citricolor* when it is grown in liquid culture. It is anticipated that this will contribute to the advancement of basic knowledge concerning the disease and perhaps provide a basis from which a long-term solution to this problem may arise.

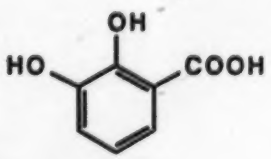
The chemistry of another phytopathogenic fungi-plant interaction was studied.

A complex of fungi[14] belonging to the genus *Ceratocystis* causes a disease of conifers known as blue stain,[15] a designation arising from the fact that the sapwood of the afflicted trees is stained a pronounced blue colour. The disease is currently responsible for the death of about 40 million trees annually in Western Canada.[16] As the blue stain fungi spread throughout and gradually encircle the sapwood of the tree, the area above the infected area becomes deprived of water. As a result, the tree rapidly wilts and dies.[17-18] Recently it has been reported[18-19] that as the blue stain develops in the sapwood, the transpiration system of the infected tree becomes restricted to the inner portions of sapwood and

eventually transpiration fails completely. Since the onset of water deprivation coincides with the appearance of the blue colour, the fungal metabolites which may be responsible for the blue colour are of particular interest. A study conducted in this laboratory on the various fungi thought to be involved in the disease has resulted in the isolation of the novel natural product ceratenolone (1)[20] and 2,3-dihydroxybenzoic acid (2)[21], from *C. minor* and *C. huntii*, respectively. 2,3-Dihydroxybenzoic acid is a known iron chelating agent[22] which gives a bright blue colour in the presence of ferric chloride. Ceratenolone also possesses the structural characteristics required for iron complexation and has been shown to form an iron chelate very readily. Thus it appears likely that the blue staining of the wood is attributable to the iron chelates of these two compounds and perhaps other, as yet unidentified metabolites of the blue stain fungi.



1



2

Microbial metabolites which function as iron transport agents are called siderophores[23]. Almost invariably these compounds incorporate *ortho*-acyl-substituted catechols (such as 2) or hydroxamic acids as the structural features which serve as ligands to coordinate the metal ion.

We decided to synthesize ceratenolone in order that its relative iron-binding capacity could be measured and its possible role in tree mortality and symptom expression studied in *in vivo* bioassays.

II. THE AMERICAN LEAF SPOT DISEASE OF COFFEE

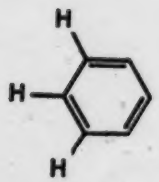
A. Metabolites of *M. citricolor*

In a preliminary investigation of the metabolites produced by *M. citricolor*, a liquid culture of the fungus was grown on 5% (w/v) bread crumbs medium in a fermentor.[24] After a growth period of 21 days, a malfunction of the temperature controller system allowed the temperature of the growth medium to reach *ca* 60°. We harvested the fungal culture by removing the mycelial mat by filtration. The culture broth was extracted with chloroform.

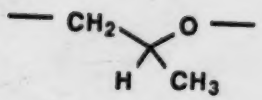
The broth extract was examined by thin layer chromatography (tlc). Amongst the several tlc spots, two showed distinctive characteristics. The more polar (component A) of the two gave a very intense fluorescence emission when ultraviolet light (uv) (254 nm) was used to illuminate the tlc plate. The other (component B) gave a yellow color upon charring with 10% sulphuric acid. Fractionation of the crude extract by centrifugal liquid chromatography (clc) followed by separation by flash chromatography[25] of the fractions containing these two compounds, allowed for their purification.

High resolution electron impact mass spectrometry (hreims) of the fluorescent compound A, obtained as colorless needles (m.p. 49-51°), indicated it possessed a molecular formula of $C_{10}H_{10}O_3$ (m/z 178, 100% intensity). Inspection of the ^1H nuclear magnetic resonance (nmr)

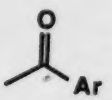
spectrum revealed that it contains a 1,2,3-trisubstituted benzene nucleus. The three aromatic protons (δ6.64, 6.83 and 7.35) possess *ortho-meta* (J = 6 and 1 Hz), *ortho-meta* (J = 7 and 1 Hz) and *ortho-ortho* (J = 6 and 7 HZ) couplings respectively. Two benzylic protons are evident as a doublet (δ2.91) coupled to a methine (sextet centered at δ4.70) which in turn shows coupling to a methyl group (δ1.50). The infrared (ir) spectrum shows an absorption for an unsaturated carbonyl group at 1672 cm^{-1}. At this stage, partial structures A, B, and C can be assembled.



A



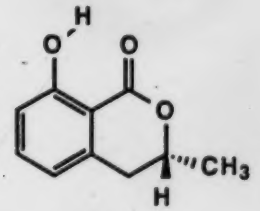
B



C

This accounts for all the atoms in the molecular formula except an oxygen and a hydrogen atom. Examination of the low field region (10-20 ppm) of the ^{1}H nmr spectrum shows a signal at δ11.0 . This chemical shift along with the absence of a normal hydroxyl absorption in the ir spectrum points to the presence of a strongly hydrogen bonded phenolic hydrogen. The structure 3, corresponding to mellein a known natural product,[26] can thus be assigned

to component A. Comparison of the spectral data (nmr, ir and ms) of 3 with that reported[27] for mellein confirms the structural assignment. A negative Cotton effect at .lambda. 257 nm (methanol), indicates our material is R-(-)-mellein.[28] Mellein has been isolated from other fungi and is known to inhibit growth of the roots of young plants.[29]



3

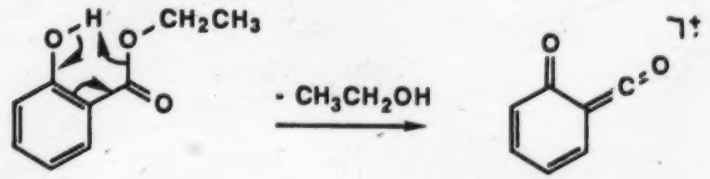
Component B gives a yellow color when its tlc spot is charred with sulphuric acid. The hreims spectrum of B indicates a molecular formula of $C_{12}H_{16}O_4$ (m/z 224, 30% intensity). Its ^1H nmr spectrum reveals that B contains two isolated ethyl groups (one as an ethoxyl group) as evidenced by the signals for two methylene groups appearing as quartets at δ4.43 and 2.89 (J = 7.1 and 7.4 Hz respectively) and two methyl groups appearing as triplets at δ1.43 and 1.20 (J = 7.1 and 7.4 respectively). An isolated methyl group is also evident by the singlet at δ2.12. The ir absorptions at 1619 and 1595 cm^{-1} in the ir

spectrum of B, together with the methine appearing at δ6.25 as a singlet in the ^{1}H nmr of B, suggest the presence of a penta-substituted benzenoid nucleus. The absorption at 1634 and 3453 cm^{-1} in the ir spectrum points to the presence of an unsaturated carbonyl group and a hydroxyl group. Two such hydroxyl groups must be present to account for the two broad signals (δ5.12 and 1.59) in the ^{1}H nmr spectrum which disappear upon treatment with deuterium oxide. The following partial structures are thus indicated.

The carbonyl group can be attached to either the methyl, the ethyl or the ethoxyl group. However, the mass spectrum of B shows a base peak at m/z 178 corresponding to the loss of an ethanol molecule. The only plausible structure that accounts for such a facile loss must incorporate the ethoxyl group as an ethyl ester. Furthermore the ethyl ester may be *ortho* to a phenolic hydroxyl group as shown in scheme 1. We thus have partial

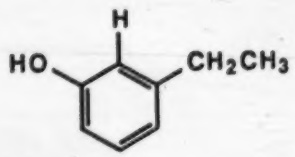
structure a.

Scheme 1 : Origin of base peak in mass spectrum of 4



a m/z 178

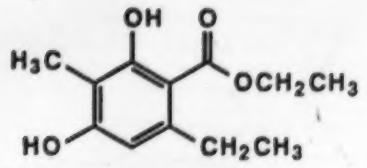
The relatively high field nmr absorption of the aromatic hydrogen indicates that it is likely located *ortho* to an hydroxyl group. In addition, irradiation of the signal at δ2.89 corresponding to the methylene of the ethyl group gave a 6.5% nOe enhancement of the aromatic signal, thus suggesting partial structure b.



b

Combining partial structures a and b, and a consideration of the probable biosynthetic polyketide

pathway, allows us to propose 4 as a possible structure for B.



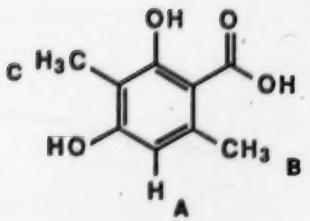
4

To our knowledge no compound possessing this structure (ester or free acid) has been reported in the literature. However, the structurally similar orsellinic acid (5) provides a model compound for comparison of the spectroscopic data[30] (see table 1). The excellent correlation observed serves to substantiate our assignment of structure 4 for compound B.

In subsequent cultures, compounds 3 and 4 were not observed. In fact, the tlc spectrum of the crude extracts was quite different. Attempts to reproduce the elevated temperature during fermentation did not reproduce these metabolites.

At this stage, we decided to investigate the metabolite production of *Mycena citricolor* in a more systematic manner. The fungus was cultured in three different ways: in a fermentor, in shake cultures, and on

Table 1. Comparison of the ^1H nmr and ir spectra of orsellinic acid 5 and compound 4.



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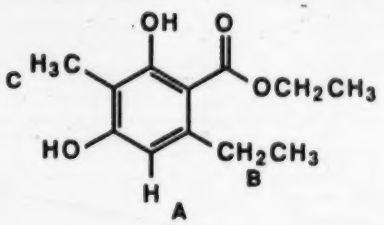


4

^1H nmr	A	6.21 s	6.25 s
	B	2.47 s	2.89 q
	C	2.01 s	2.12 s
ir		3400	3453
		1635	1634
		1612	1619
		1596	1595
		1490	1320
		1266	1278
		1097	1110

solid media. The medium used was potato dextrose, a medium routinely used in our laboratory to grow a variety of fungi. The cultures were grown for different lengths of time with varying amounts of yeast extract added. Table 2 summarizes the results of the study. The metabolite spectra from the three growth methods were virtually the same when analysed by tlc. An antibiotic bioassay of mycelium and broth extracts against several bacteria and fungi afforded negative results.

Table 2. Metabolite production using different growth methods.

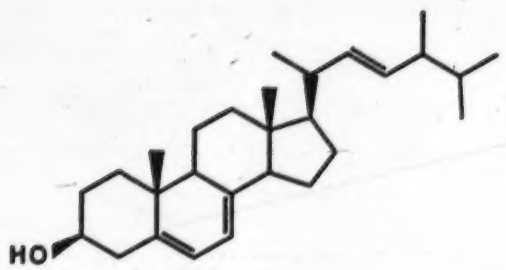
growth time	fermentor	shake cultures		plates	
	PDB	PDB/yeast	PDA	PDA/yeast 0.2%	PDA/yeast 5%
weeks	mg/L	mg/L		mg/10 plates	
2	28	290	-	-	
3	-	150	-	116	
4	21	-	42	81	152
5	-	-	-	80	
6	-	-	-	37	

Each fungal culture was harvested and the broth was extracted as before. The major components of the organic extracts were separated by flash chromatography and

preparative tlc. The extracts were found to consist mainly of fatty acids and steroids (ergosterol (6), ergosterol peroxyde (7), and cerevisterol (8)). The structure of each compound was assigned by comparison of spectral data with that of an authentic sample. The identity of each steroid was further confirmed by comparison of its tlc behaviour with that of an authentic sample.

We reasoned that our strain of *Mycena* might have degenerated since it was maintained on synthetic medium for a prolonged period of time. A fresh culture of *M. citricolor* was obtained from Costa Rica. However, when it was grown in shake cultures, it too gave negative tests in the antibiotic bioassay, and a the tlc spectrum of the extract was similar to that obtained previously. In order to obtain a very virulent isolate of *M. citricolor*, plant pathologist Dr. J.P.Tewari suggested that we infect coffee leaves with the fungus and reisolate it from the infected leaves. Such a reisolated culture was grown in shake cultures under the same conditions as before. The broth extract displayed a tlc spectrum which was different from that obtained previously. The extract (ethyl acetate) was found to show some antibiotic activity against *Proteus vulgaris*, *Staphylococcus aureus* and the fungus *Candida albicans*. The mycelium extract showed a weaker antibiotic activity.

In order to study the effect of the medium on the observed antibiotic activity several different media were



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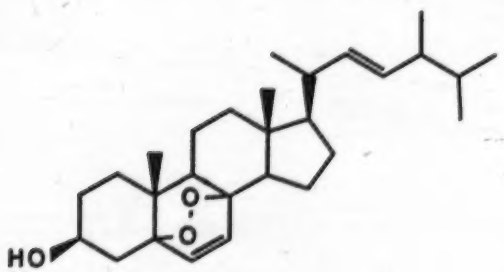

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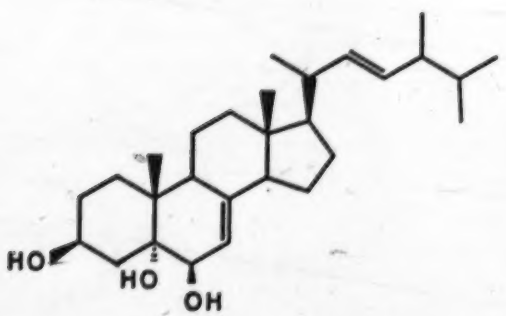


8

$CH_3(CH_2)_{14}COOH$

9

$CH_3(CH_2)_4(CH{:}CH)CH_2(CH{:}CH)(CH_2)_7COOH$

10

investigated. Media containing potato dextrose / yeast extract, V8 juice, coffee extract, and a chemically defined medium were innoculated with fungus freshly isolated from coffee leaves. After 6 to 8 weeks, the fungal cultures were harvested as before. The broth and mycelial extracts were subjected to antibiotic testing. The greatest bioactivity was observed for the ethyl acetate broth extract of still cultures grown on potato dextrose containing 2% yeast extract.

This "active" extract was found to be very complex upon examination by tlc using various solvent systems. Different techniques were used to effect preliminary fractionation, including droplet countercurrent chromatography (dccc),[31] acid - base - neutral separation, and flash chromatography. None of the fractions obtained by any of these fractionation methods showed the activity previously observed in the crude extracts. The bioactivity observed for the crude extracts was found to be irreproducible even though the tlc spectrum of the extracts was constant from growth to growth. Nevertheless, we investigated and identified the metabolites of this culture of *M. citricolor*.

The extract of the fermentation broth of still cultures of *M. citricolor* afforded as the major component a crystalline compound $C_{21}H_{32}O_5$, m.p. 202-205°, for which we suggest the name citricolic acid (11). The molecular formula was established by a combination of chemical

ionization mass spectrometry (cims), which indicated a molecular weight of 364, and high resolution mass spectrometry (hrms) which provided the molecular formula ($C_{21}H_{30}O_4$) for the peak at m/z 346 (M^+-H_2O).

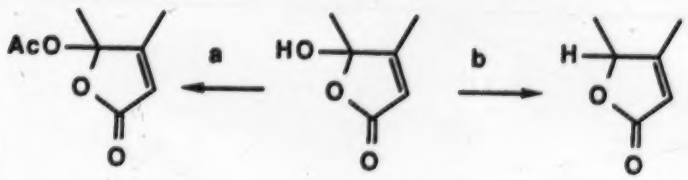
Infrared (ir) spectroscopy shows the presence of a carbonyl group with a strong absorption band at 1740 cm^{-1} as well as absorption at 3440 cm^{-1} characteristic of -OH groups. The ^{13}C nmr spectrum displays a signal at δ172.5 ppm which along with the ir data suggests the presence of an α,β-unsaturated ester or lactone. The ultraviolet spectrum (uv) (MeOH) has an absorption maximum at 217 nm (ε = 9900) which shifts reversibly to about 255 nm upon addition of a few drops of dilute NaOH. These data are consistent with the presence of the lactol form of an α,β-unsaturated γ-keto acid (partial structure A).

A

This partial structure accounts for the ^{13}C nmr signals at δ172.5, 171.9, 117.6 and 105.3, as well as for the ^1H nmr peak at δ5.74. Citricolic acid forms a

monoacetyl derivative 15 when treated with pyridine/acetic anhydride and is reduced when treated with sodium borohydride, affording an α,β-unsaturated γ-lactone 14.

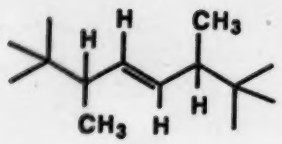
Scheme 2 : Formation of acetyl- and deoxy- citricolic acid



a) Py / Ac_2O
b) $NaBH_4$ / MeOH

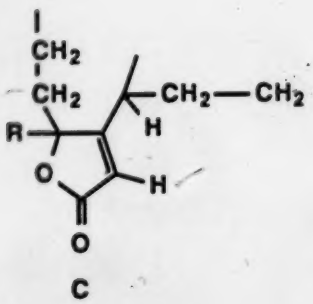
The 400 MHz ^1H nmr spectrum and extensive decoupling studies reveal several other features present in the molecule. Olefinic proton signals at δ5.61 and 5.48 are mutually coupled (J=15.5 Hz), indicating the presence of a *trans* disubstituted double bond. Each of these is further coupled to allylic methines at δ2.41 and 2.13, respectively. Each methine hydrogen is coupled to a methyl group (δ1.07 and 1.03, respectively), but not further coupled. Partial structure B, bearing a quaternary center at each end, is consistent with these data.

The olefinic proton at δ5.74 is coupled to a methine hydrogen at δ3.40 and this in turn is coupled to a methylene group (δ1.76 and 1.62). The signal at δ1.76 is further coupled with another methylene group (δ2.01 and 1.64). The nature of the fragment attached to the γ carbon



B

of the lactol ring is revealed by the ^1H nmr of the $NaBH_4$ reduction product C(R=H). The γ-proton signal at δ4.64. is coupled to a methylene group (δ2.41 and 1.6) and also shows long range coupling to another methylene group (δ1.89 and 1.73). The methylene signals are mutually coupled. These data are consistent with the expanded partial structure C (R=OH) for citricolic acid.



C

The mass spectrum of citricolic acid shows an intense peak at m/z 59 corresponding to C_3H_7O. This along with

methyl singlets at δ1.20 and 1.16 in the ^{1}H nmr spectrum and one at δ72.5 in the ^{13}C nmr, indicates the presence of a carbon atom bearing two methyl groups and a hydroxyl group (partial structure D). The carbon spectrum also shows a singlet at δ84.4, which indicates that the fifth oxygen is present as part of a tertiary alcohol group (partial structure E). A quaternary methyl is apparent at δ0.71 in the ^{1}H nmr spectrum (partial structure F).

Analysis of the mass spectrum shows that major peaks occur for C_{21}, C_{18} and C_{12} ions. The loss of $C_9H_{17}O$ is the most pronounced fragmentation of citricolic acid and its acetylated and reduced derivatives. This suggests that the $C_9H_{17}O$ unit is present as a side chain on a C_{12} skeleton. Ozonolysis of citricolic acid provides a compound (16) which has lost a six carbon unit, the C_{12} segment remaining intact, confirming this observation. The hreims base peak has a formula C_9H_{16} and can arise via a McLafferty type fragmentation by hydrogen tranfer from the C_{12} portion of

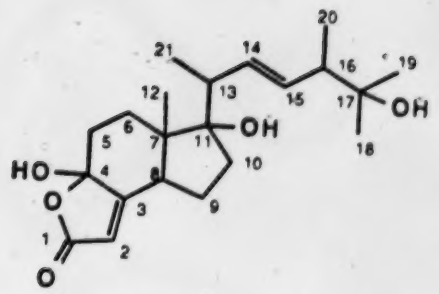
the molecule with the subsequent loss of H_2O. This fragmentation, which is similar to that observed with some β-phenyl alcohols[32], is illustrated in partial structure G, an expansion of partial structure B. Hydrogenation of citricolic acid provides a dihydro compound, 18, which does not show this type of fragmentation.

Scheme 3 : Origin of base peak in mass spectrum of 11

G m/z 124

The facile loss of $C_9H_{17}O$, arising from the cleavage of a bond α to an hydroxyl group and allylic to an olefinic bond, is also explained by G. The structural unit G is reminiscent of the ergosterol side chain and accounts for the part structures D and E. Partial structures C and F account for the remaining eleven carbons. The various units are logically assembled to give three possible structures for citricolic acid, 11, 12, and 13.

Structure 13 may be rejected after consideration of the following nOe experiment on citricolic acid. Irradiation of the singlet methyl group at δ0.7 gives a 1.4% nOe of the proton at δ5.74. This observation cannot


20
21
14
16
19
12
13
15
17
OH
OH
18
5
6
7
11
HO
4
8
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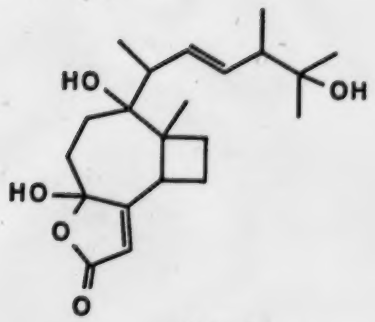
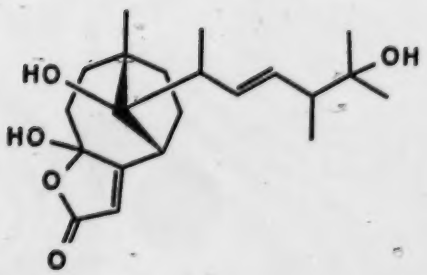
3
9
1
2
O

11


HO
OH
HO
O
O

12


HO
OH
HO
O
O

13

be accommodated by structure 13. Structure 12, on the other hand, may be rejected since the observed fragmentation pattern is not consistent with this structure. Compounds with a four membered ring containing two adjacent methylene groups usually lose a molecule of ethylene in the mass spectrum. Bearing in mind a possible biogenetic relationship with the common fungal metabolite ergosterol, structure 11 for citricolic acid is favored.

In order to fully assign the ^{1}H nmr spectrum of citricolic acid, a COSY experiment[33] was carried out. As shown in figure 2 (bold line; shown for only one side), the side chain spin system, previously derived by selective decoupling experiments, is apparent (see fig. 1). Also apparent, (light line fig. 2) as previously derived, is the coupling between the olefinic proton (δ5.74) and the methine at δ3.40, which is further coupled to two protons at *ca* δ1.6. It is not possible to unambiguously assign the coupling partners of this methylene (see fig. 4).

Figure 1. Side chain spin system

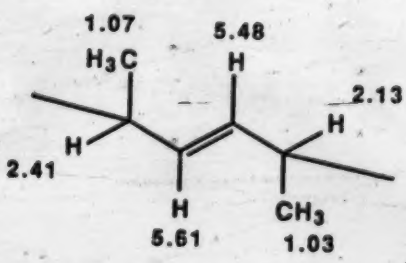


Figure 2. COSY Spectrum of citricolic acid

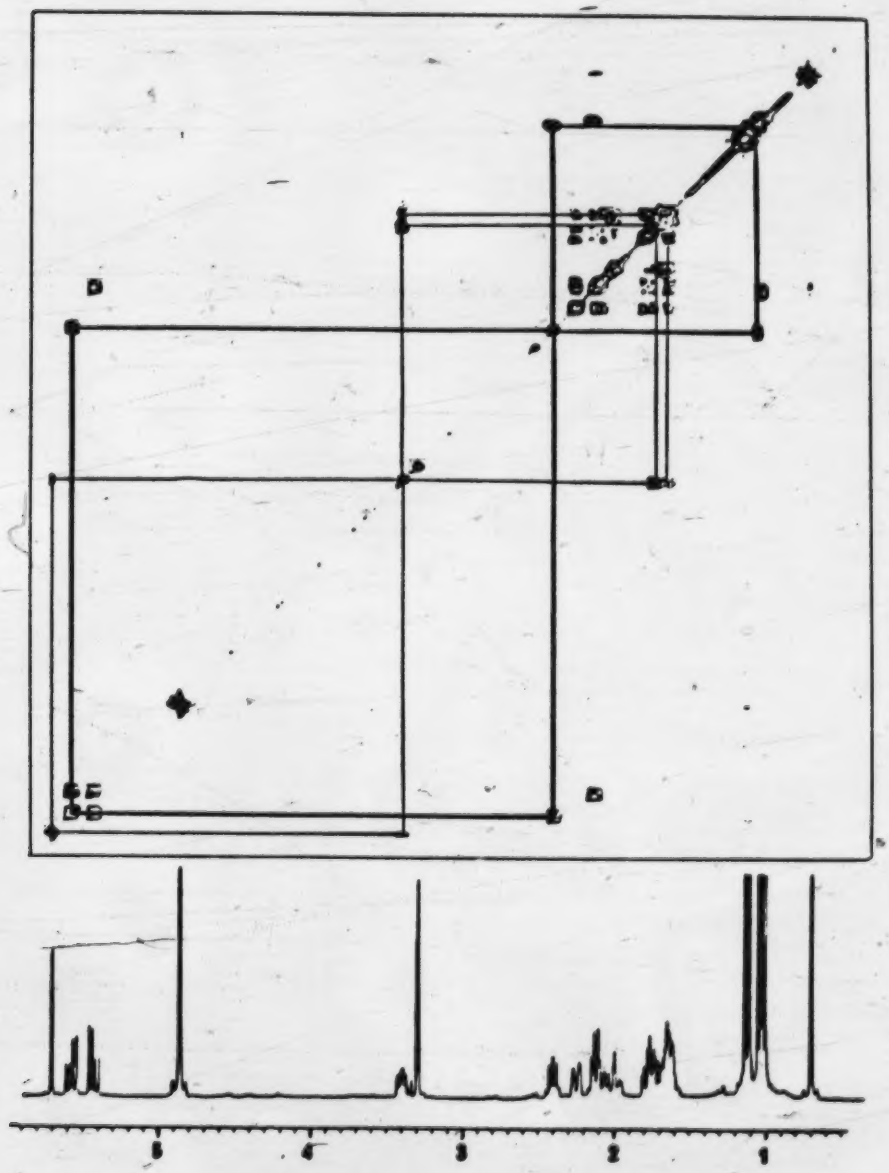


Figure 3. Expansion of COSY spectrum of citricolic acid

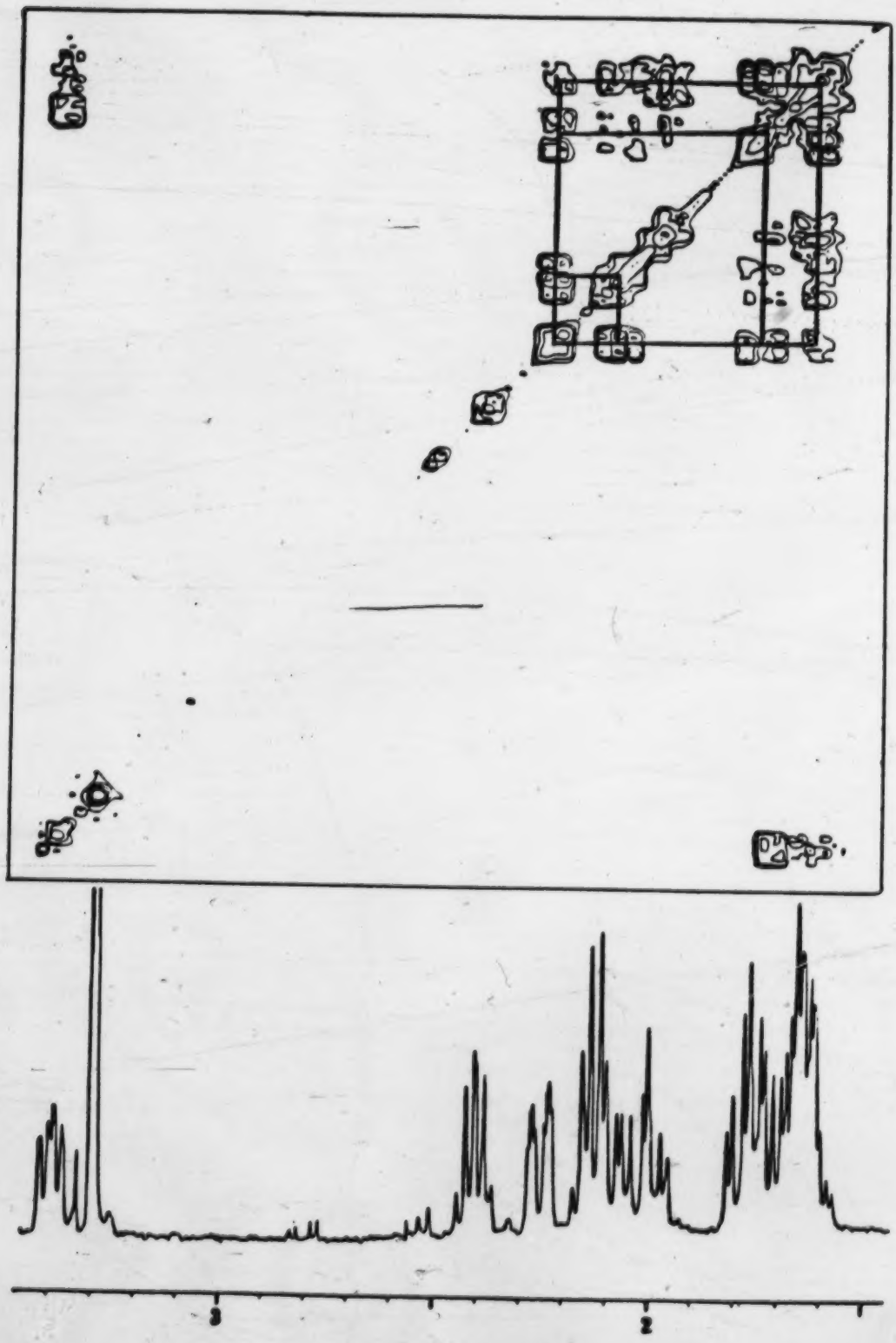
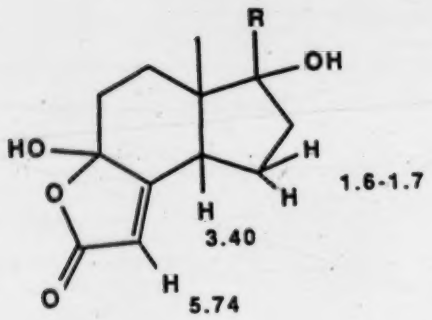
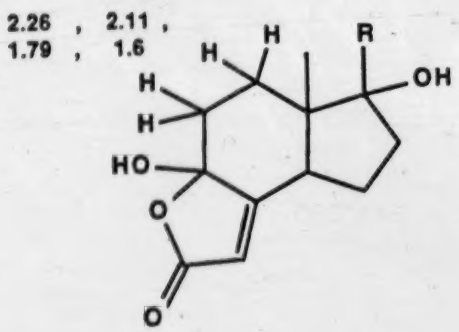


Figure 4. Spin system A



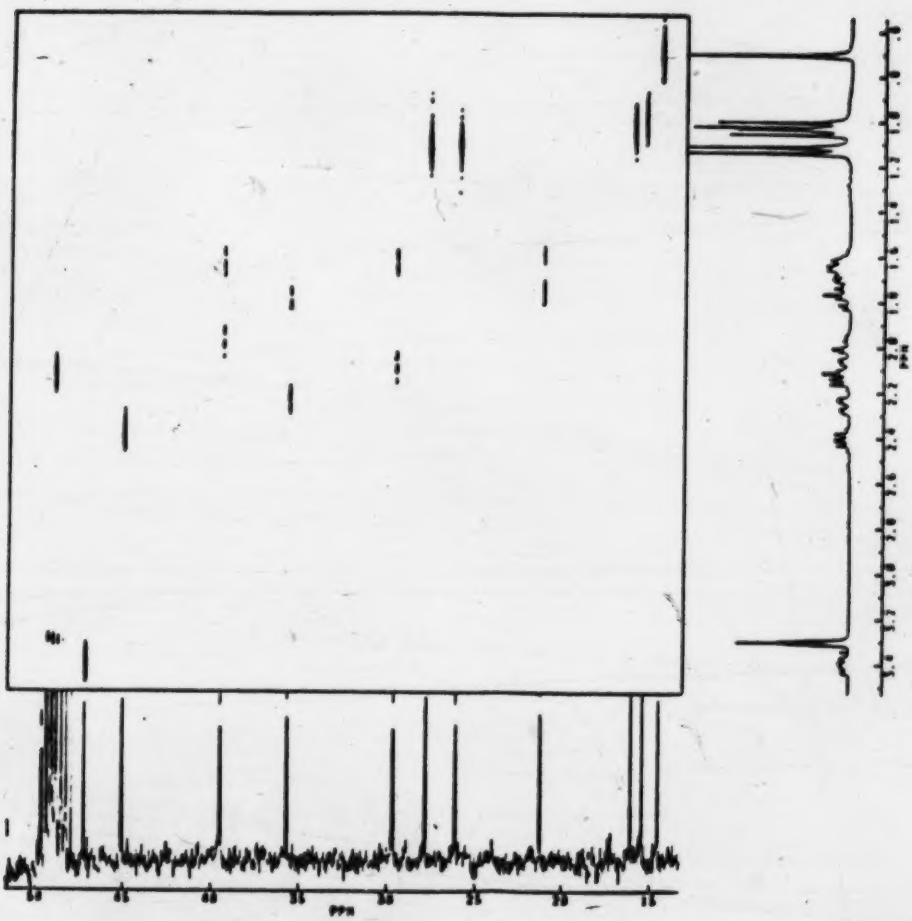
As shown in figure 3, the proton at δ2.26 couples to the protons at *ca* δ2.0, 1.8, and 1.6 . Since at least three protons of this spin system have chemical shifts other than 1.6, they cannot be assigned to protons present in the B ring and must therefore form part of the A ring system, as shown in figure 5.

Figure 5. Spin system B



It should be possible to simplify the assignment problem with the aid of a $^{13}C/^{1}H$ correlated spectrum[34] and this spectrum is shown in figure 6. The signals

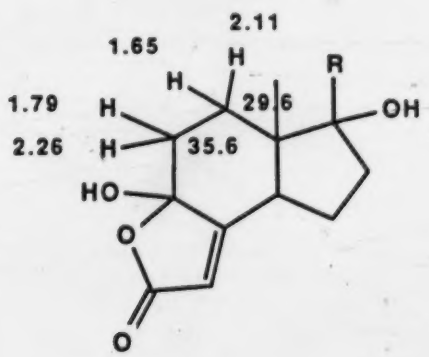
Figure 6. ^{1}H / ^{13}C correlation spectrum



corresponding to methylene groups in the δ1.5 - 2.0 region of the ^{1}H nmr were observed.

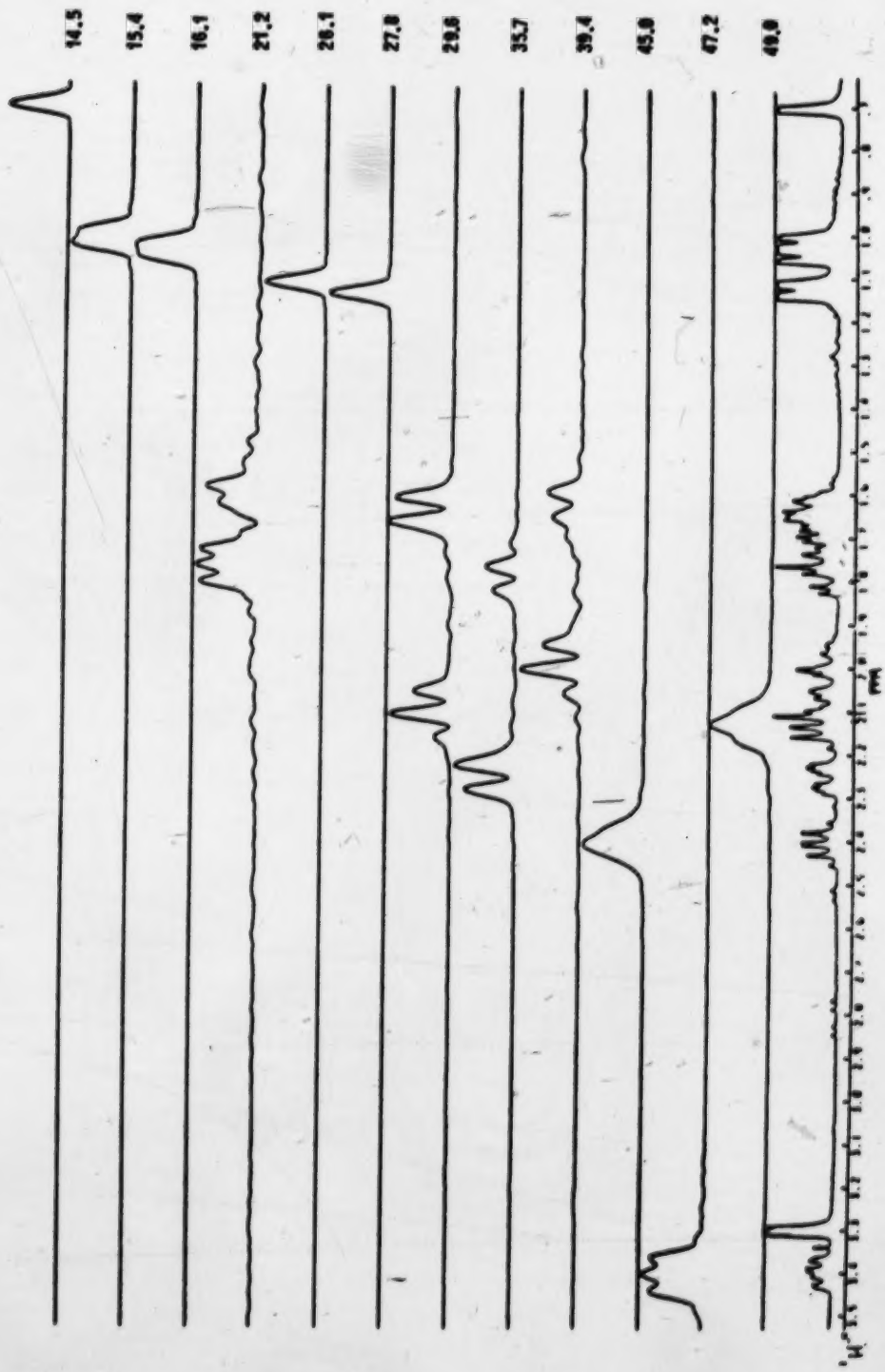
Slices showing the vertical amplitude of the ^{1}H spectrum for each of the ^{13}C signals are shown in figure 7. The isolated ^{1}H signal at δ2.26 is correlated to the ^{13}C signal at 35.65. Its geminal coupling partner appears at δ1.79. Since the proton at δ2.26 is also coupled to protons at δ2.11 and 1.64, the adjacent methylene group must be one which has a signal at δ29.62 correlated to those two proton signals. This assignment is depicted in figure 8.

Figure 8. ^{1}H and ^{13}C assignments for spin system B



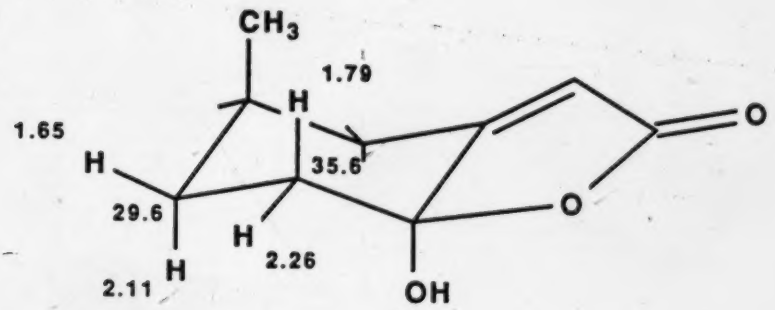
Furthermore, the protons at δ2.26 and 1.64 exhibit one large geminal coupling constant whereas the protons at δ2.11 and 1.79 show *two* large coupling constants as evidenced by their appearance as triplets on the correlation lines. The protons whose signals have the additional large coupling constant must therefore be axial and mutually coupled according to the Karplus rule[35]

Figure 7. Vertical cuts from the ^{1}H / ^{13}C correlation spectrum



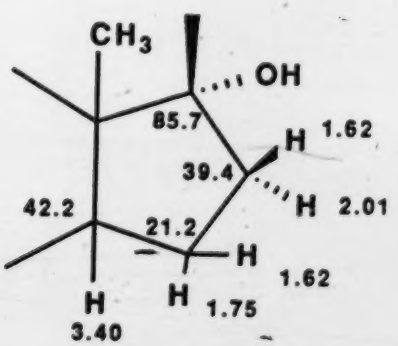
(figure 9).

Figure 9. ^{1}H and ^{13}C assignments for spin system B (stereo.)



The methine at δ3.40 is coupled to protons at δ1.62 and 1.75. The methylene carbon which shows correlation to these two protons is that at δ21.20. The remaining methylene with proton signals at δ2.01 and 1.62 correlated to the ^{13}C signal at δ39.42 (figure 10). The lowest field methylene proton at δ2.01 must be *cis* to the hydroxyl group at C-11.

Figure 10. ^{1}H and ^{13}C assignments for spin system A



Complete assignments for the ^{1}H and ^{13}C nmr spectra of compound 11 are given in Table 3. Treatment of citricolic acid (11) with ethereal diazomethane provides the keto ester 17. Ozonolysis of deoxycitricolic acid and reductive ($NaBH_4$) workup gives the diol 16.

The assignment of the configuration of the C-7 methyl and C-11 side chain of citricolic acid was partly based upon comparison of the carbon-13 nmr spectrum of 11 with those of appropriate model compounds.

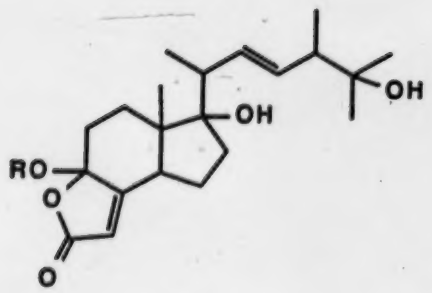
Compounds 19a-c were obtained by degradation of vitamin D.[36] The ^{13}C nmr chemical shifts assignments of these compounds are presented in Table 4 (columns 2-4). The effect of substitution of an hydroxyl substituent for a hydrogen at C-17 or C-25 on the chemical shift of neighboring carbon atoms is shown in columns 5 and 6 respectively. When both C-17 and C-25 bear an hydroxyl substituent, the chemical shift of neighboring carbon atoms may be calculated by combining the perturbation values obtained for the mono-hydroxylated derivatives. These calculated values are presented in column 7.

Comparison of the observed chemical shifts for ergosterol (6)[37] and citricolic acid (11) with those calculated for 17,25-dihydroxyergosterol (20) (using the shift parameters obtained in table 4) are presented in Table 5.

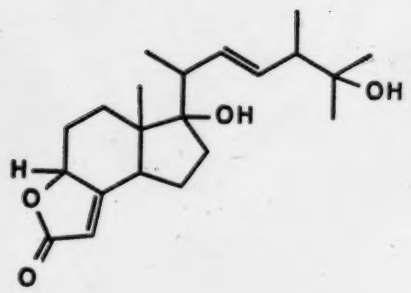
The correlation between the predicted values for the model compound 17,25-dihydroxyergosterol (20) and

Table 3 . Nuclear Magnetic Resonance Data for Citricolic Acid (11)

	^{13}C	^{1}H mult. (J,Hz)	
1	174.68	-	
2	113.06	5.74	d 2.0
3	173.71	-	
4	102.30	-	
5	35.64	1.79	ddd 13.5, 13.5, 4.5
		2.26	ddd 13.5, 4.0, 2.0
6	29.62	1.65	m
		2.11	ddd 14.0, 14.0, 4.5
7	54.84	-	
8	47.18	3.40	ddd 12.0, 7.0, 2.0
9	21.19	1.62	m
		1.76	dd 12.0, 7.0
10	39.42	1.64	m
		2.01	dd 14.0, 11.0
11	85.75	-	
12	14.53	0.71	s
13	65.01	2.41	dq 7.5, 7.0
14	133.53	5.61	dd 15.5, 7.0
15	134.97	5.68	dd 15.5, 7.0
16	49.04	2.13	dq 7.0, 7.0
17	73.35	-	
18	26.10	1.11	s
19	27.79	1.14	s
20	15.43	1.03	d 7.0
21	16.06	1.07	d 7.0



11 R = H
15 R = Ac



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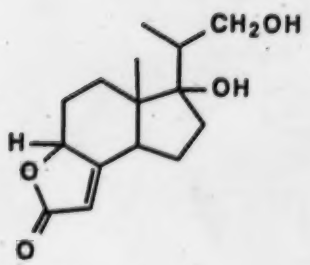

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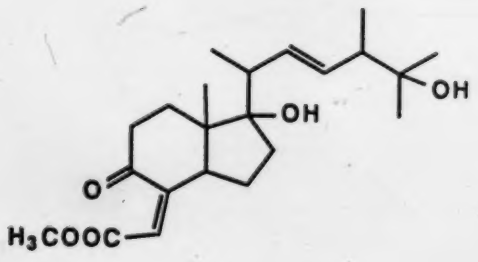

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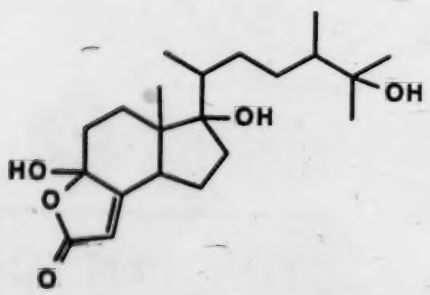

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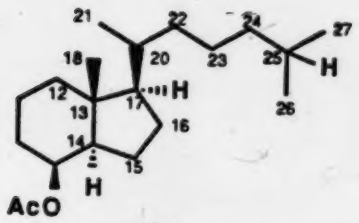
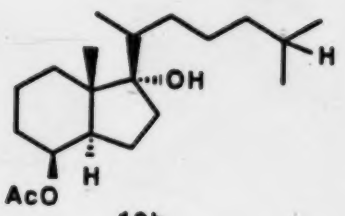
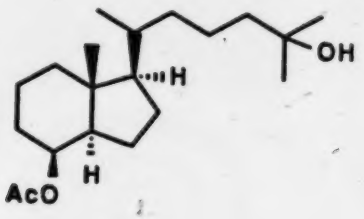
Table 4. Calculation of the effect of OH substitution on ^{13}C shifts



19a



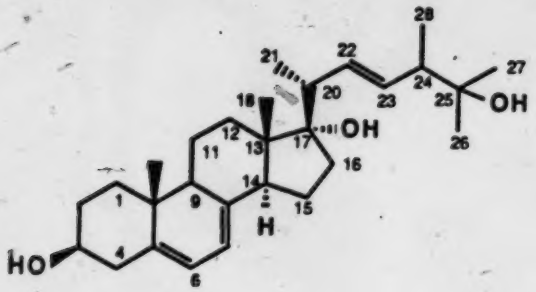
19b



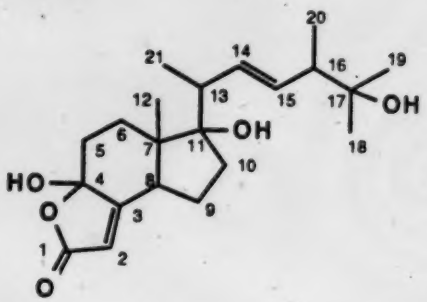
19c

C no	19a	19b	19c	δb-δa	δc-δa	
12	40.25	33.40	40.60	-6.85	0.35	-6.50
13	42.10	47.60	41.95	5.50	-0.15	5.35
14	51.55	46.05	52.80	-5.50	1.25	-4.25
15	22.80	21.90	22.65	-0.90	0.15	-1.05
16	27.15	37.95	27.25	10.80	0.10	10.90
17	56.75	86.20	56.90	29.45	0.15	29.60
18	13.10	15.85	13.60	2.75	0.50	3.25
20	35.45	39.20	35.30	3.75	-0.10	3.65
21	18.70	13.95	18.60	-4.75	-0.10	-4.85
22	36.10	32.40	36.40	-3.95	0.25	-3.70
23	23.90	25.65	20.90	1.75	-3.00	-1.25
24	39.65	39.40	44.50	0.25	4.85	5.10
25	28.05	28.05	70.95	-	42.90	42.90
26	22.60	22.50	29.20	-0.10	6.60	6.50
27	22.80	22.80	29.35	-	6.50	6.50

Table 5. Comparison of the ^{13}C nmr of 11 with the calculated nmr of 20



20

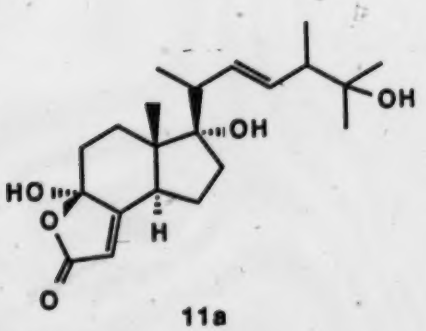


11

C no	6	(20)	C no	11
12	28.3	21.8	6	29.6
13	43.0	47.4	7	54.8
14	54.6	50.3	8	47.2
15	23.1	22.0	9	21.2
16	39.4	50.3	10	39.4
17	56.0	85.6	11	85.7
18	11.8	15.0	12	14.5
20	40.5	44.2	13	45.0
21	19.4	14.6	21	16.0
22	132.2	128.5	14	133.5
23	136.0	134.7	15	134.9
24	43.0	48.1	16	49.0
25	33.2	76.1	17	73.3
26	19.7	26.2	18	26.1
27	21.0	27.5	19	27.8
28	17.4		20	15.4

citricolic acid (11) is quite good. The overall agreement between "predicted" and observed values suggests the relative stereochemistry of 11 and 20 to be the same, i.e. *trans* ring junction of the C-D rings and *cis* relationship between the C-7 methyl group and the side chain.

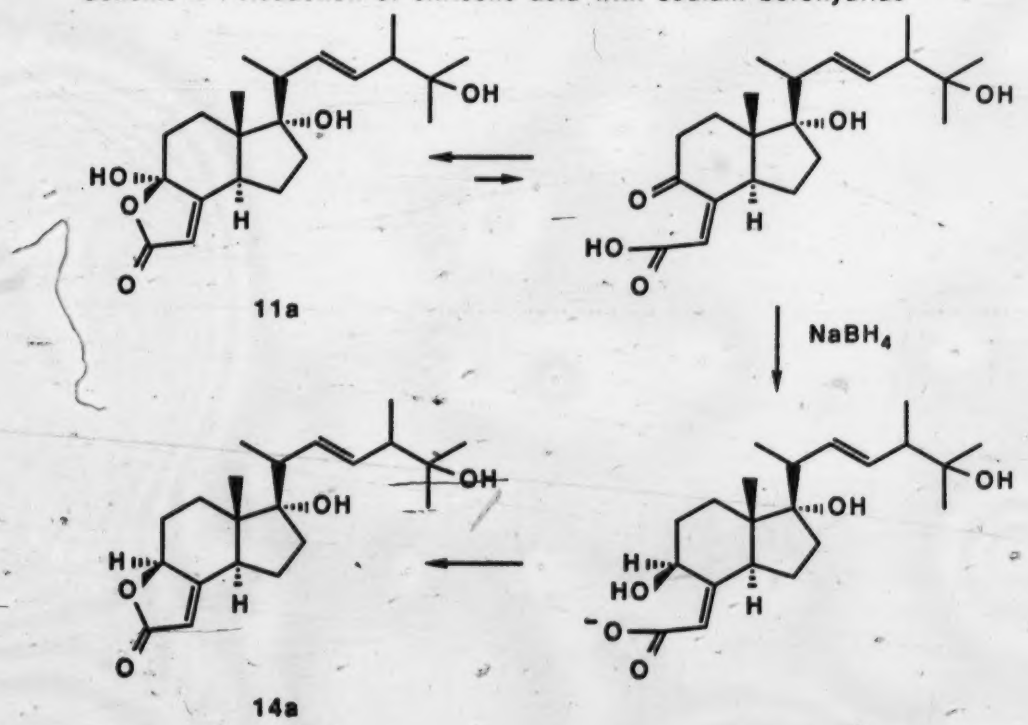
The relative stereochemical assignment was confirmed by a nuclear Overhauser effect (nOe) experiment. Irradiation of the methyl group at C-7 results in a 3.8% signal enhancement of the proton at C-13 as well as a 1.4% nOe to the proton at C-2. This confirms the β side chain configuration. Inspection of molecular models reveals that the C-4 hydroxyl group must be in an axial orientation, the six-membered ring in a chair conformation with a *trans* ring fusion to the 5-membered ring. This is the only conformation in which the C-2 hydrogen is in proximity to the C-7 methyl group. Therefore, the stereochemistry must be as depicted in 11a.



11a

Reduction of citricolic acid with sodium borohydride gives deoxycitricolic acid (14). One expects the hydride to be delivered from the alpha face of 11 because of the beta axial C-7 methyl group. Thus deoxycitricolic acid should have the stereochemistry shown (see scheme 4). This is indeed the observed stereochemistry for 14 as evidenced by the 2 Hz coupling between H-2 with both H-4 and H-8 in the ^{1}H nmr spectrum of 14.

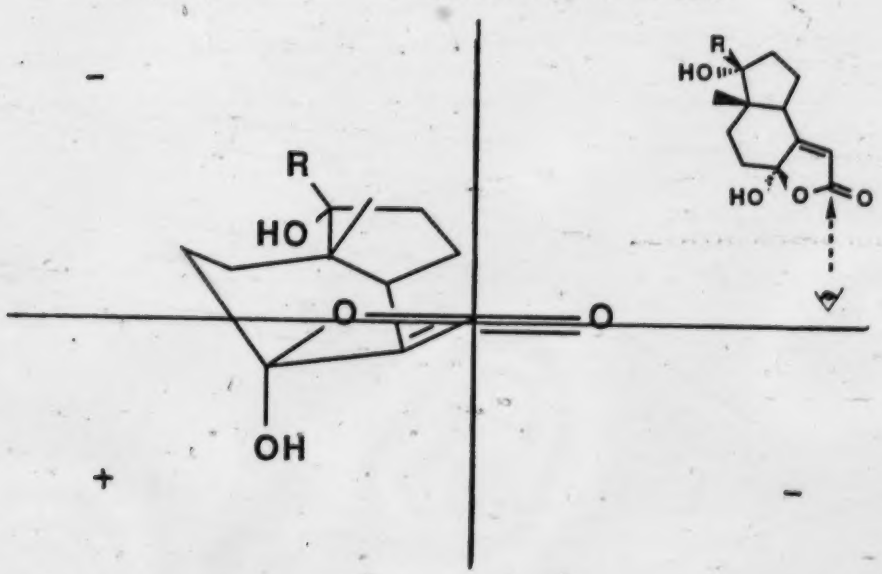
Scheme 4 : Reduction of citricolic acid with sodium borohydride



The absolute configuration of citricolic acid (10) was determined using the lactone sector rule.[38] The rule is empirical and similar to the ketone octant rule,[39] but with inverted quadrants. The sign of the Cotton effect can be

predicted from the superimposition of lactone quadrants to a conformational drawing of a given absolute configuration. Figure 11 shows a partial drawing of 11 for the absolute configuration as seen along the C-1 - C-2 bond axis with the molecule in the rear quadrants. Since the chirality of the lactone ring determines the sign of the Cotton effect one would predict a positive Cotton effect for this configuration.

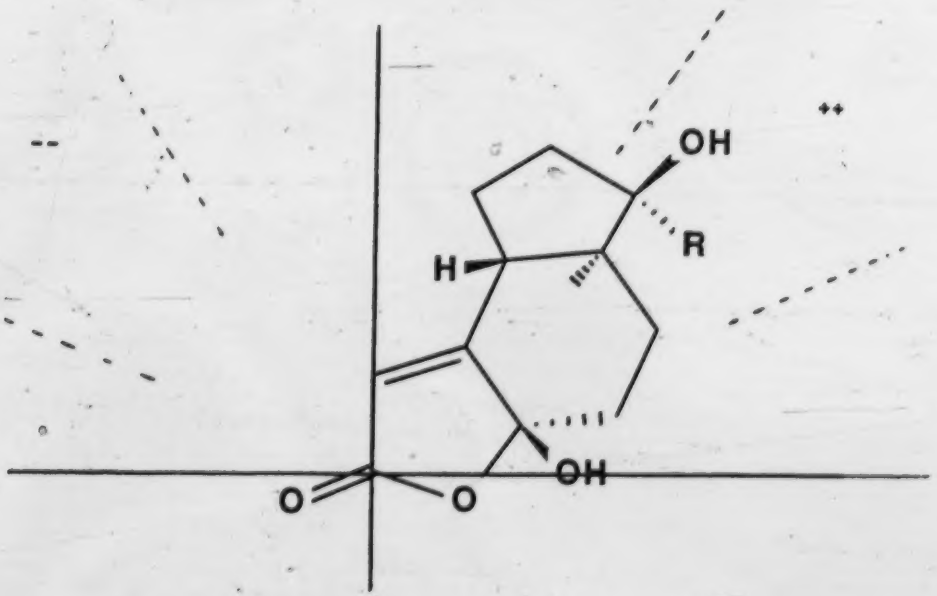
Figure 11. Lactone sector rule applied to citricolic acid



Another empirical method which employs circular dichroism data requires superimposing the two ketone octants corresponding to the two "ketones" of a lactone.[40] One thus derives the following octants, where the zones

denoted (++) and (--) receive the same sign contribution from both ketone octants.

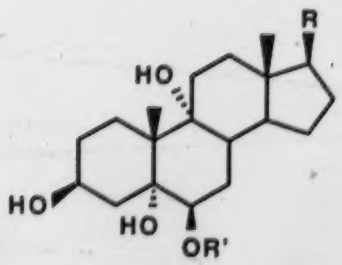
Figure 12. Lactone sector rule (II) applied to citricolic acid



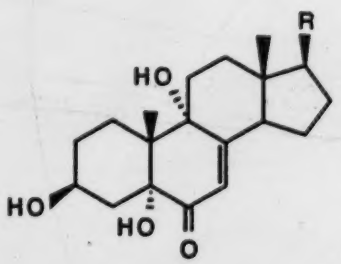
Application of this method to citricolic acid, as shown in figure 12, also predicts a positive Cotton effect for this absolute configuration. Measurement of the circular dichroism of citricolic acid shows a strong

positive Cotton effect at 243 nm . We therefore conclude that the absolute configuration of citricolic acid is that shown in 11a.

The structural similarity between citricolic acid (11) and ergosterol (6) was mentioned above. Both ergosterol and cerevisterol (8) have been isolated from the mycelium extract of *M. citricolor*. This suggests that ergosterol (6) may be the biosynthetic precursor of citricolic acid (11). Fungal steroids oxygenated at both C-6 and C-9 have been reported. For example, the three steroids 21, 22, and 23, were isolated from *Polyporous versicolor*, the European wood rotting fungus.[41]



21 R' = H
22 R' = CH_3



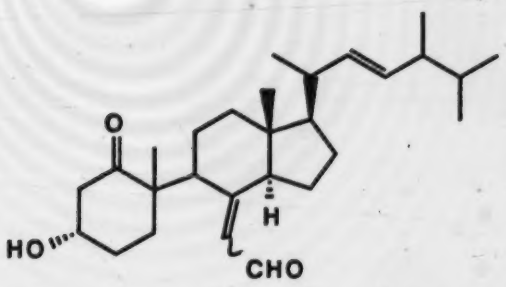
23

Hydroxylation of the steroidal side chain at C-25 is precedented;[42] however, to the best of our knowledge, no compound with the ergosterol side chain bearing a C-25 hydroxyl substituent is known.

It is possible that citricolic acid is derived by oxidative cleavage of a ring B oxygenated precursor 25,

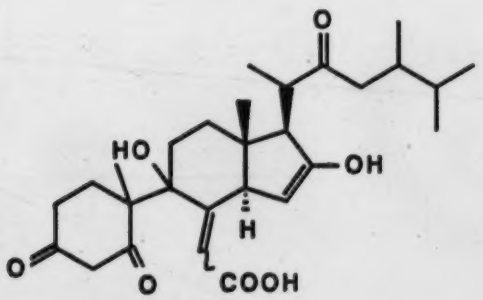
similar to 23 as illustrated in scheme 5.

An early reported degradation product of cerevisterol, the C-5, C-6 dihydroxylated derivative of ergosterol, is aldehyde 27.[43]



27

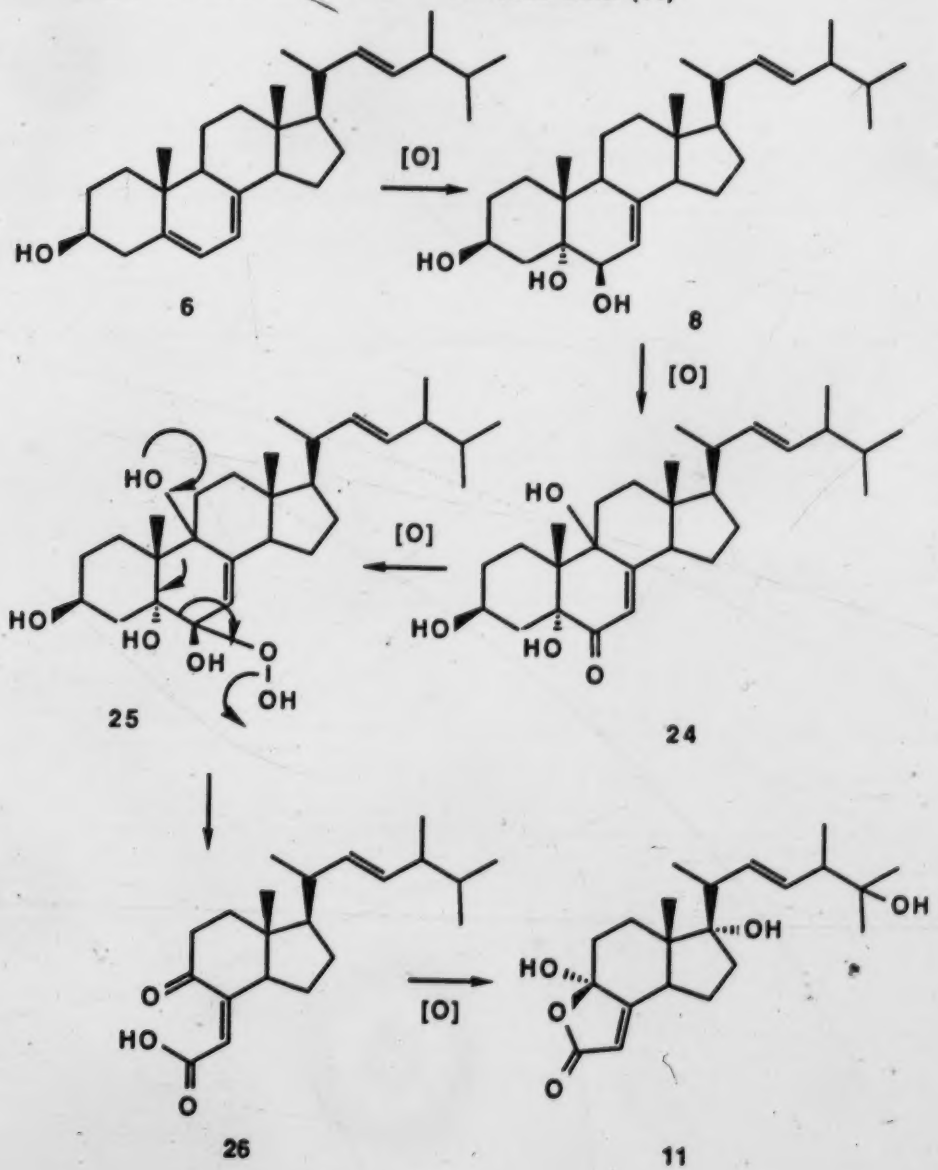
More recently Christiani isolated a compound derived from ergosterol, by degradation with ergosterol-oxydase. He proposed structure 28 for this degradation product.[44]



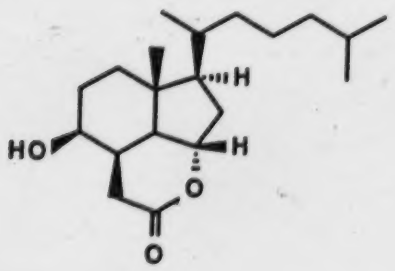
28

To the best of our knowledge, the carbon skeleton found in citricolic acid (11) has not been observed

Scheme 5 : Possible biogenesis of citricolic acid (11)



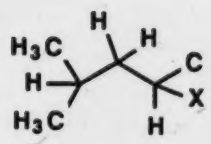
previously in a naturally occuring compound. Very recently, the similarly degraded steroid 29, which lacks the methyl at C-24 (steroid numbering) and which possesses a different oxidation pattern, has been isolated from a sea hare.[45]



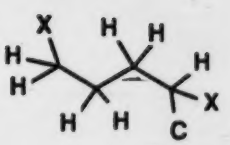
29

A compound with molecular formula $C_{11}H_{18}N_2O_2$ was also isolated from the culture broth. It was identified as the cyclic dipeptide L-prolyl-L-leucyl anhydride (30) on the basis of the following information. The molecular weight of 210 was confirmed by cims. Its ir spectrum showed the presence of amide carbonyl absorptions (1670 and 1635 cm^{-1}). Its ^1H nmr spectrum displays a signal at 5.79 ppm (bs) attributable a secondary amide proton. Selective decoupling experiments establish the presence of spin systems A and B.

The mass spectral fragmentation pattern (scheme 6) of compound 30 agrees with the assigned structure. Comparison of the spectral data of an authentic sample of

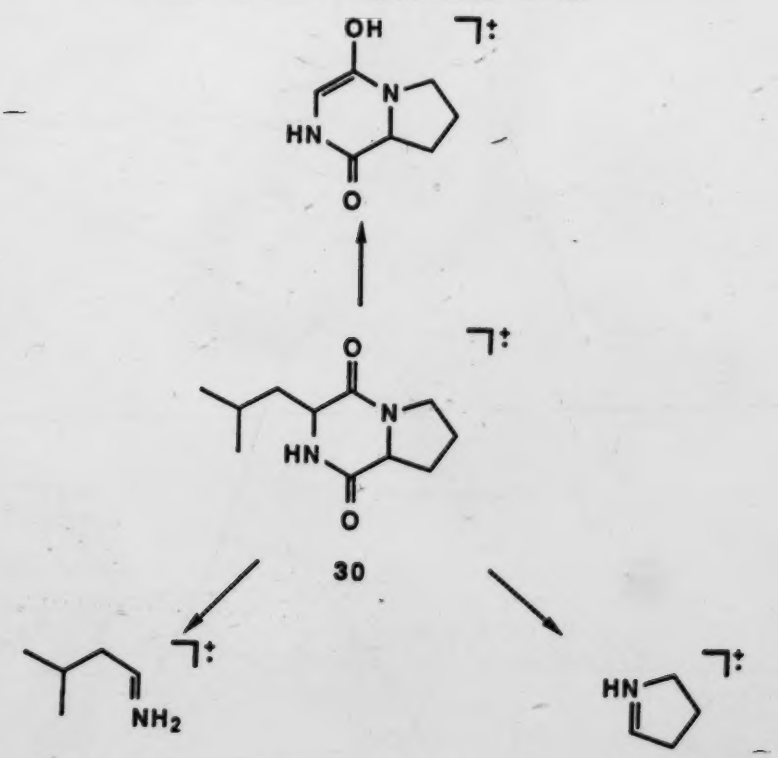


A



B

Scheme 6 : Mass spectral fragmentation of 30

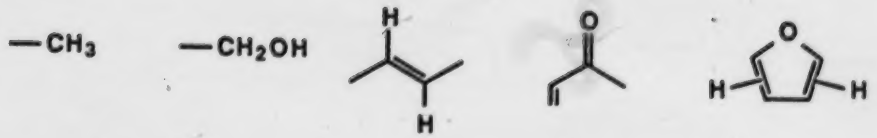


L-prolyl-L-leucyl anhydride with that of 30 confirmed their identity.

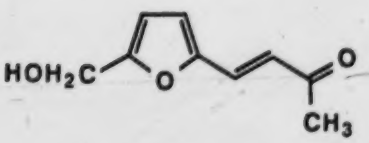
This cyclic dipeptide, (as well as other diketopiperazines) has been isolated from a number of sources, i.e., culture filtrates of several bacteria,[46] as an insecticidal metabolite of *Asperillus orchaceus* and *Oospora destructor* (pathogenic fungi of silk worms).[47] It was also reported recently from the blue stain fungi *C. clavigera*.[48]

A strongly uv-active compound (R_f 0.35, methanol - chloroform (7:93)) was isolated from the broth extracts. It has a molecular formula of $C_9H_{10}O_3$ (hreims), and hence 5 unsaturations. The base peak in the mass spectrum indicates the loss of CH_2OH from the molecular ion. The ^1H nmr spectrum displays a methyl group (δ2.36, s), an isolated methylene bonded to oxygen (δ4.68, s), and *trans* olefinic protons (δ6.65, 7.30, J = 15.5 Hz). The ir spectrum shows a conjugated carbonyl absorption band at 1682 cm^{-1}. These data leave C_4H_2O to account for the remaining 3 unsaturations and this is suggestive of a disubstituted furan.

Further examination of the ^1H nmr spectrum reveals that the proton signals from the olefinic fragment exhibit no further coupling and must therefore be adjacent to quaternary centers. The furanoic protons have chemical shifts in agreement with those expected for C-3 and C-4 of the furan ring. Protons at C-2 and C-5 would be further



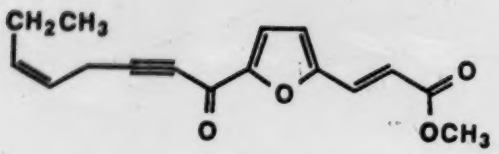
downfield. This information allows us to propose 31 as a possible structure for this compound.



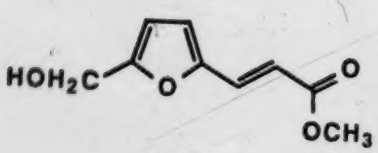
31

A similarly substituted furan, compound 32 has been isolated from the roots of the broad bean (*Vicia faba*).[49] Its stucture was proven by synthesis *via* intermediate 33.

Compound 33 has very similar spectral properties (uv, ir, nmr) to the compound which we isolated, confirming structure 31. Furan 34 has also been isolated from the broth of *M.citricolor* and may be a biosynthetic precursor

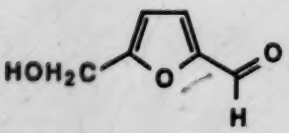


32



33

to 31. This is the first report of this natural product (31) in the chemical literature.



34

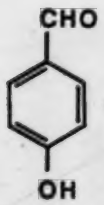
Several other minor metabolites were isolated and identified. Their structural assignment was unambiguously established by comparison with authentic samples. These metabolites, compounds 35 to 39, are shown below.

It is noteworthy that 34 and 39 have also been isolated from the broth extract of several fungi grown in this laboratory using potato dextrose broth extract as the growth medium. At times they have been isolated in very significant amount (up to *ca* 100 mg / 10 L of medium). It is therefore likely that they arise *via* degradation of sugars in the growth medium and may not be produced in nature by the fungus.

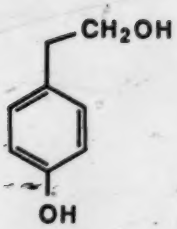
B. Leaf spot bioassay

When the metabolites isolated from *M.citricolor* were applied to coffee leaves, no disease-like symptoms were observed. The crude organic extracts also failed to show any activity. However, the aqueous broth (before and after extraction with ethyl acetate) was found to induce tissue lesions very similar to that observed on fungi infected leaves.

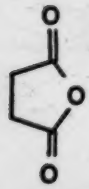
At about this time, Tewari and Rao observed crystals formed on agar plates cultivates of *M. citricolor*. These crystals were identified as calcium oxalate.



35



36



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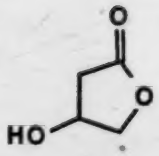

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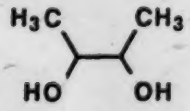


39

Subsequently, oxalic acid was found to be present in the broth by precipitation with calcium chloride and identification of the precipitate as calcium oxalate. When a 0.2% aqueous solution of oxalic acid was applied to a coffee leaf disease-like symptoms appeared. This suggests that oxalic acid may act as a calcium sequestering agent and that this results in tissue degradation. We subjected other polycarboxylic acids which could also form chelates with calcium to the leaf spot bioassay. All polycarboxylic acids tested produce similar symptoms on coffee leaves.

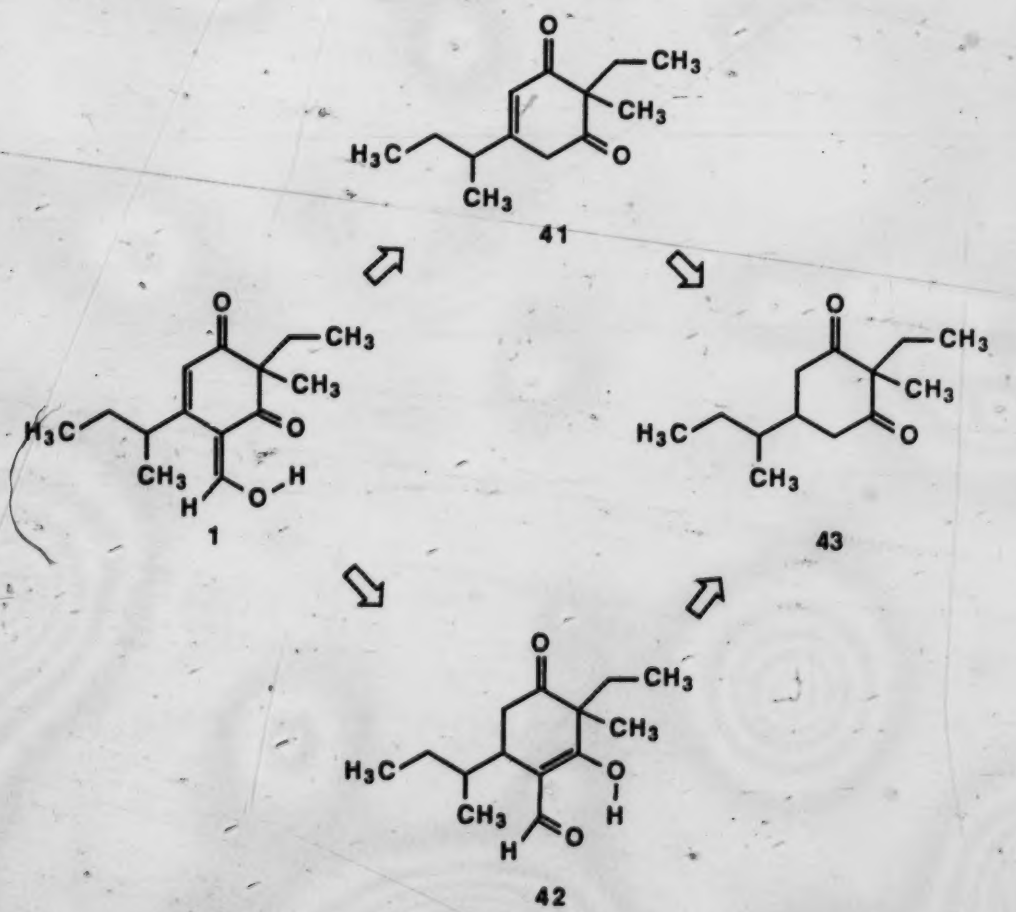
We therefore believe that the disease expression on coffee by *M.citricolor* is possibly caused by organic acids, such as oxalic acid or others, and that their mode of action is either or both chelation of calcium / magnesium, or lowering of the tissue pH. Work is currently in progress to elaborate on this hypothesis.

III. THE BLUE STAIN DISEASE OF LODGE POLE PINES

A. Synthesis of ceratenolone

In our approach to the synthesis of ceratenolone (1), we envisioned diketone 43 to be a key intermediate. Two steps, a formylation and a dehydrogenation, would then be required. Ceratenolone could be prepared from either enone 41 or α-formylketone 42, both of which can be derived from the common precursor 43.

Scheme 7 : Proposed synthesis of ceratenolone

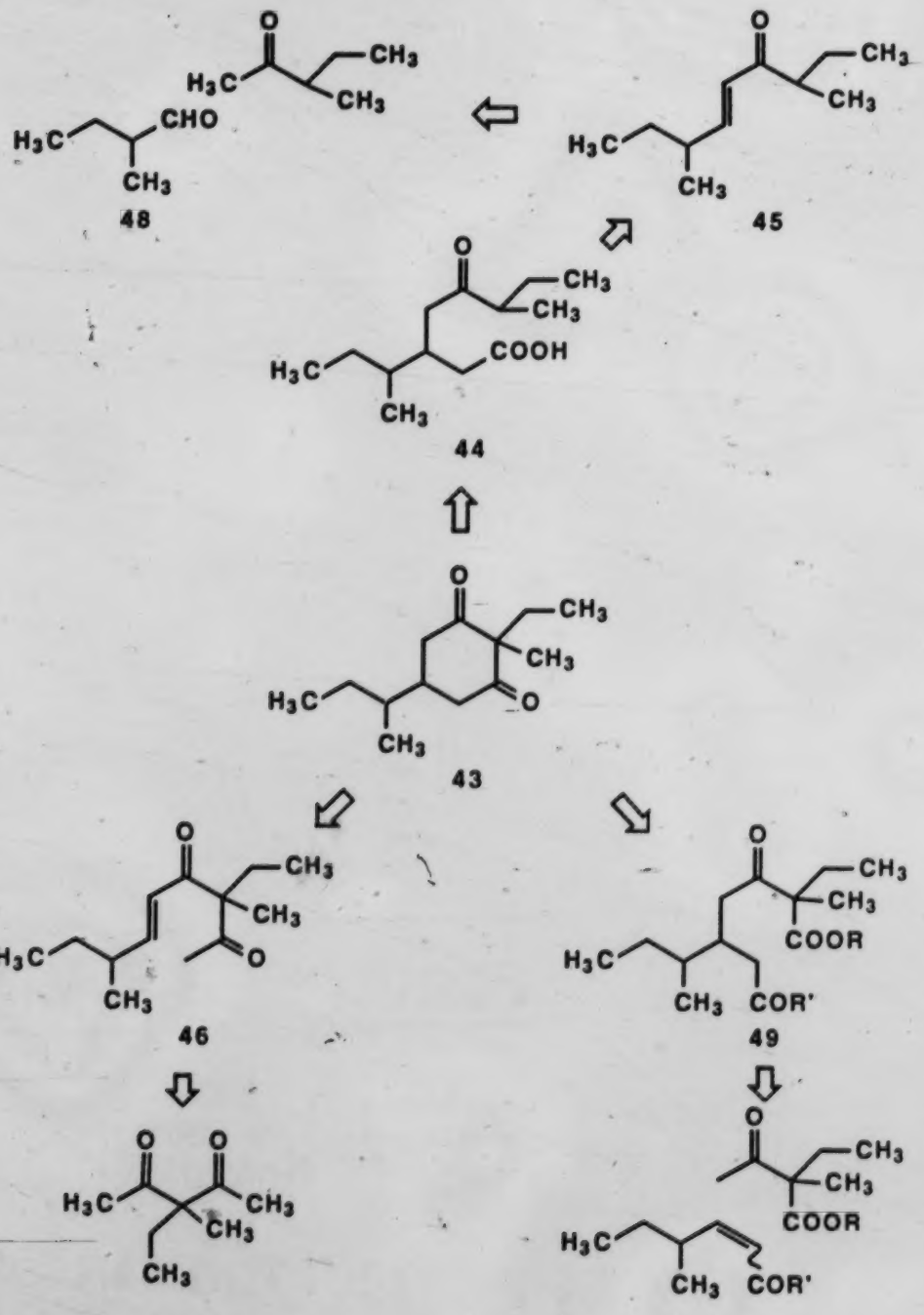


Compound 43 may be approached *via* several different routes. The "acyclic" approach is shown in scheme 8. It involves either an intramolecular acylation (with 44 or 49), or an intramolecular Michael addition (with 46). Each of these intermediates should in turn be readily available from simple precursors.

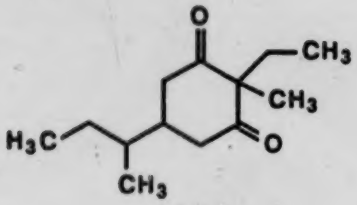
The "cyclic" approach is outlined in scheme 9 and involves the use of a 2-substituted 1,3-cyclohexanedione (52 or 53). For intermediate 52, the additional substituent at C-2 may be introduced by alkylation. For intermediate 53, the substituent may be introduced by Michael addition to a suitable Michael acceptor ,e.g., a vinyl sulphone. In practice, alkylation is the most direct approach but is generally inefficient. The Michael addition of β-diketones proceeds efficiently. However, it will require additional transformations to convert the Michael adduct to an ethyl group. Cyclohexanediones 52 or 53 can be prepared from either an acyclic (54 or 55) or cyclic (58) precursor. Both the acyclic and cyclic precursors should be readily available from simple starting materials.

Amongst the various routes available for the preparation of 43, we decided to direct our initial efforts to the synthesis of 52, as outlined in scheme 10. This appeared to be the most direct and simple route to 43 since alkylation of 52 with iodomethane would then afford 43. Of the two strategies available for the preparation of 52,

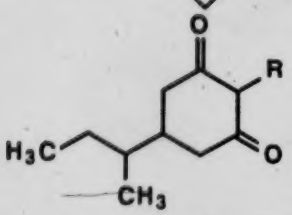
Scheme 8 : Acyclic approach to 43



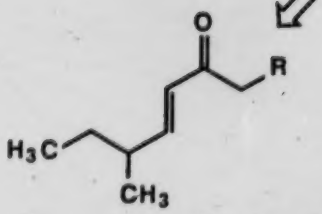
Scheme 9 : Cyclic approach to 43



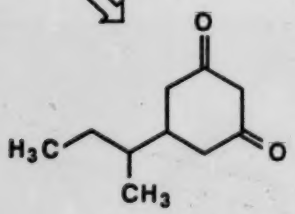
43



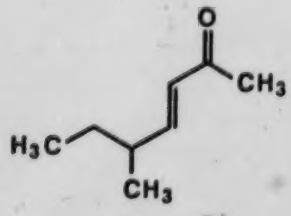
52 R = CH_2CH_3
53 R = CH_3



54 R = CH_2CH_3
55 R = CH_3



58



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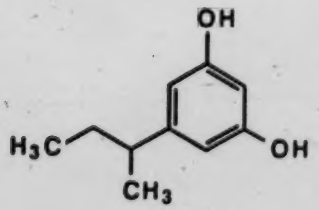
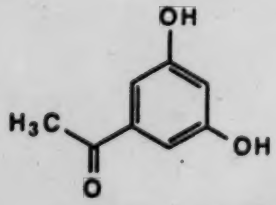
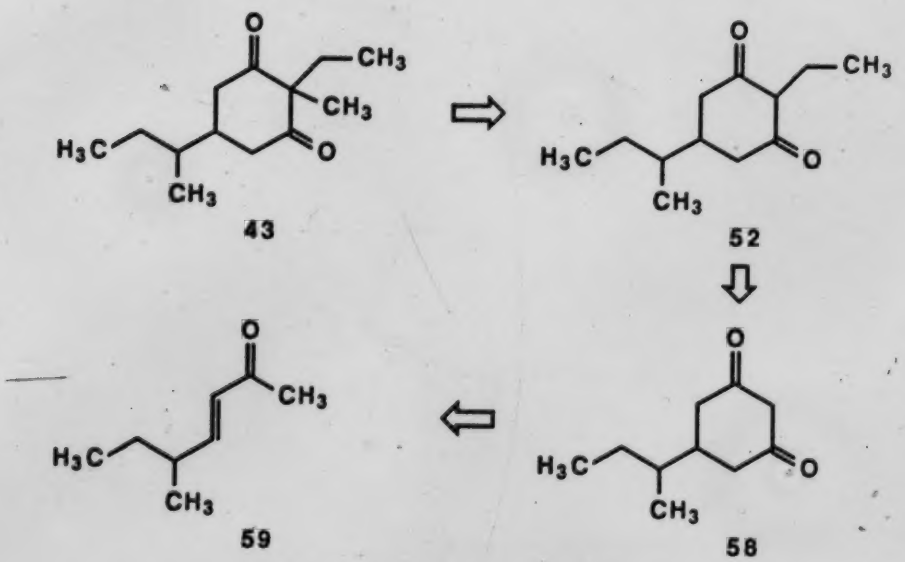


60



i.e., either from enone 54 or dione 58, we decided to opt for the latter, cyclic approach. This route is experimentally the simplest, and will allow for a large scale synthesis to be developed.

Scheme 10 : Initial approach to 43

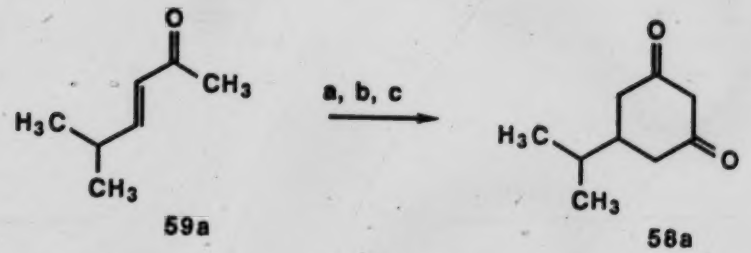


Previously the isopropyl analog of 58, 58a, has been prepared from an enone precursor as shown in scheme 11.[50]

We therefore decided to prepare dione 58 in an analogous way.

The attempted preparation of enone 59 by aldol condensation of acetone with 2-methyl butyraldehyde (48) produced a complex mixture of products. This result is not surprising since it is well known that acetone readily

Scheme 11 : Synthesis of an analog of 58

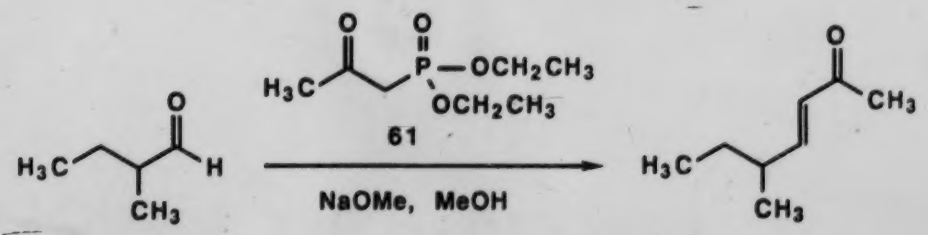


59a 58a

a) $CH_2(COOCH_2CH_3)_2$, NaOEt, EtOH
b) KOH, H_2O c) HCl, H_2O

undergoes self-condensation and that aldols involving acetone usually proceed in modest yields at best.[51] For example, enone 59a was prepared in this way albeit in only 20% yield (Scheme 11).[52]

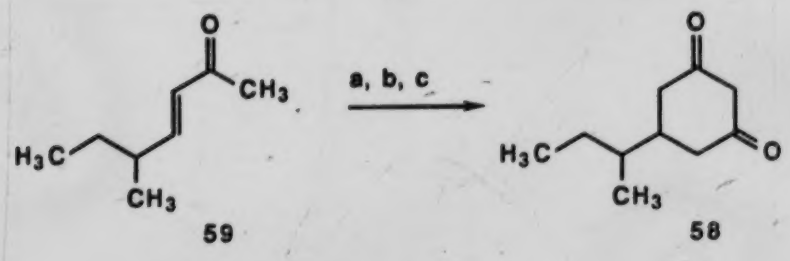
Enone 59 may also be prepared with the Wittig reaction, using Emmons reagent 61 as an acetone equivalent. Indeed when compound 61 (prepared from chloroacetone)[53] was reacted with aldehyde 48, one product, enone 59, was obtained in very good yield (93%).



48 59

The ^1H nmr spectrum of 59 displays a singlet (δ2.33) for the methyl ketone and two methine protons (δ6.02 and 6.66). The multiplicity of the methine signals (doublets of doublets with one J = 16.0 Hz) establishes the *trans*-configuration for the C-3, C-4 double bond.

Further treatment of enone 59 with diethyl malonate in the presence of sodium ethoxide in refluxing dry ethanol, followed by hydrolysis (KOH, H_2O) and decarboxylation (HCl) afforded the desired cyclohexanedione 58 in 69% yield.

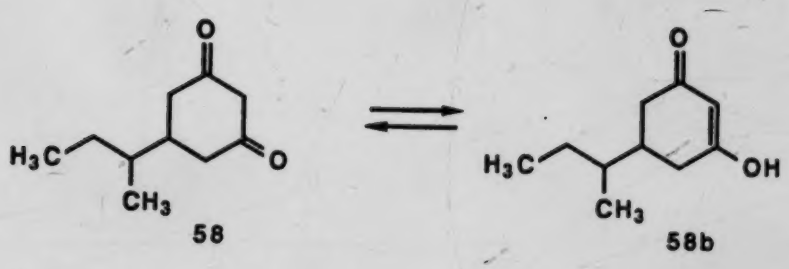


59 58

a) $CH_2(COOCH_2CH_3)_2$, NaOEt, EtOH
b) KOH, H_2O c) HCl, H_2O

The ^1H nmr spectrum of 58 shows signals corresponding to a *ca* 1:1 mixture of tautomers. The signals at δ8.7 and 5.48 correspond to the hydroxyl and olefinic protons of the enol form 58b respectively, while the singlet at δ3.40 corresponds to the methylene protons of the keto form 58.

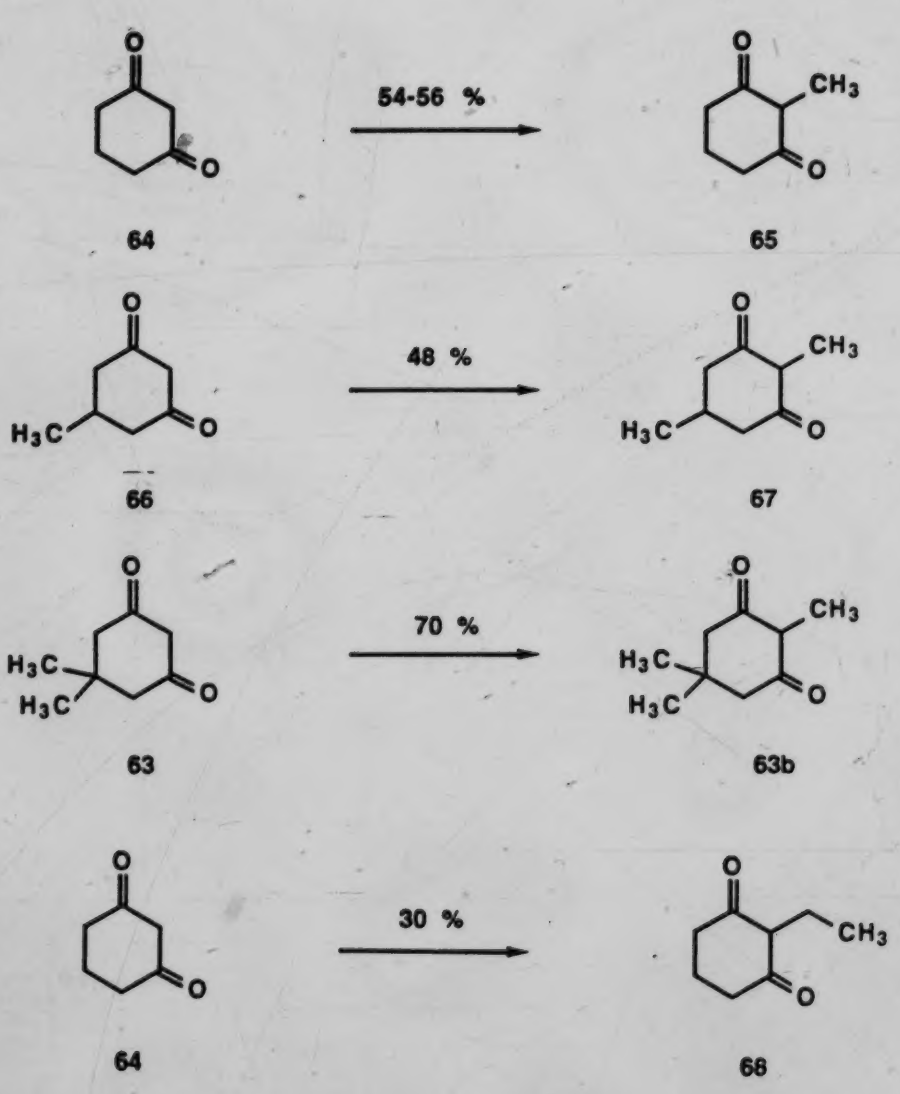
Having prepared the desired dione 58 in satisfactory yield, the problem became that of introducing an alkyl substituent, either methyl or ethyl, at C-2 (Scheme 9). C-Alkylation of β-diketones has been a long standing



58 58b

problem in organic synthesis.[54] Recent methods of C-alkylation give good yields with *acyclic* β-diketones,[55] but these methods still produce low yields with *cyclic* β-diketones.[56] For example, methylation of cyclohexane-1,3-dione (64) proceeds in 54-56% yield,[57] while 5-methylcyclohexane-1,3-dione (66) affords 48% yield of product[58] and dimedone (63) is methylated in 70% yield.[59] As well, C-alkylation with an ethyl group proceeds in an even lower yield. For example, ethylation of cyclohexane- 1,3-dione gives 68 in *ca* 30% yield.[60]

One alternative to simple alkylation of a β-diketone is the method developed by Piers and Grierson for incorporation of an alkyl group between two carbonyl groups.[61] The method, outlined in scheme 12, involves the Birch reduction of a resorcinol methyl ether 69, followed by generation of the anion using *tert*-butyllithium, and subsequent quenching with an alkyl iodide to give 70. Subsequent hydrolysis affords high yields of 2-substituted



64 65

66 67

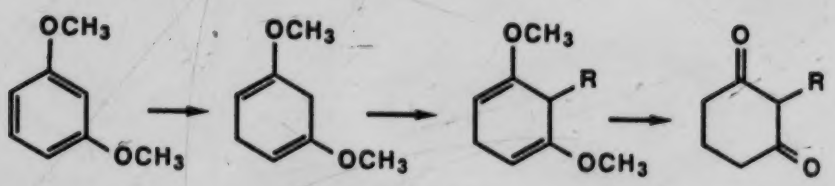
63 63b

64 68

cyclohexane-1,3-diones (71). This alternate alkylation sequence is however, not applicable to our synthetic route.

Two consecutive alkylations are necessary in the transformation of 58 to 43. As the introduction of an ethyl group is more difficult than a methyl group, we decided to introduce the ethyl group in the first

Scheme 12 : Method of Piers and Grierson

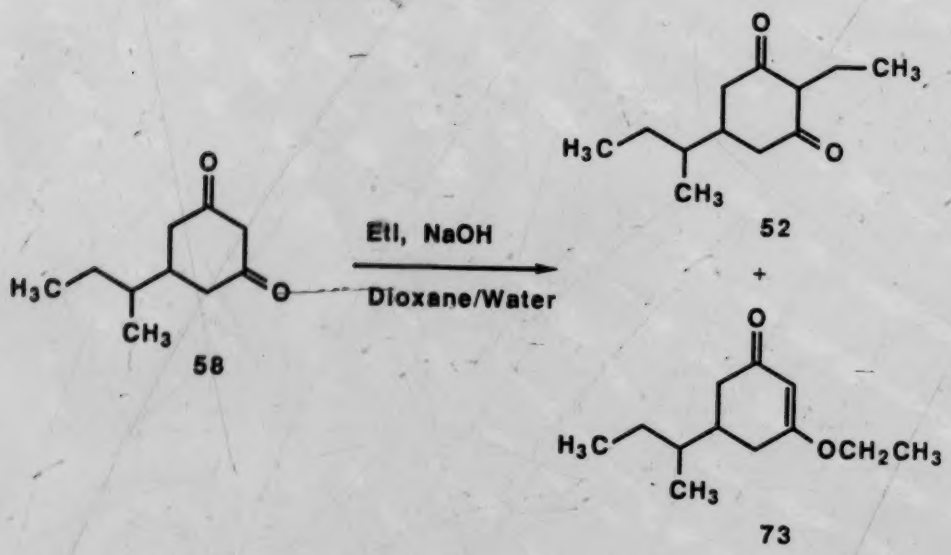


69 70 71

alkylation.

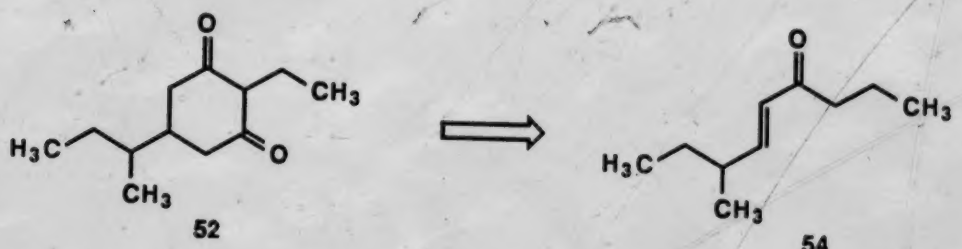
In order to optimize the ethylation conditions, we undertook a study of the alkylation of cyclohexane-1,3-dione (64) with iodoethane. Treatment of 64 in water - dioxane with sodium hydroxide, followed by treatment with iodoethane afforded the C-alkylated dione 68 in a modest yield (42%, lit.(56b): 30%). This result, plus the possibility of recycling the O-alkylated portion, was encouraging. However, when the same conditions were applied to dione 58, two major products were isolated. The desired C-alkylation product 52 was obtained in very poor yields (9-19%). The ^{1}H nmr spectrum of 52 shows the presence of a tautomeric mixture of diastereomers. A broad singlet at δ7.2 and a quartet at δ2.30 correspond to the hydroxyl proton and the methylene of the ethyl group of the enol form 52 while two triplets at δ3.25 and 3.28 correspond to the diastereomeric α-methine protons of the keto form. The other product, obtained in 35-45% yield,

was the O-alkylated dione 73. The ^1H nmr spectrum of 73 displays an olefinic methine (δ5.38) and an isolated ethyl group (quartet at δ4.92 and triplet at δ1.39). Hence, O-alkylation was the predominant reaction product.



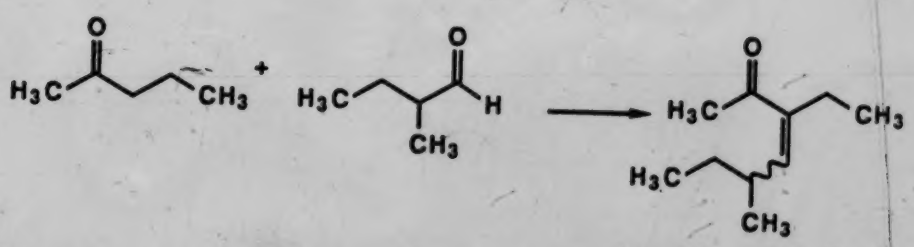
Attempts to increase the yield of compound 52 by employing prolonged reaction times, or excess iodoethane, resulted in increased amounts of the O-alkylation product 73 and polar decomposition products. The very low yield of C-alkylation product makes the proposed recycling process a very inefficient one. We therefore decided to abandon this sequence.

Incorporation of the ethyl group in the dione molecule *i.e.*, dione 52, avoids some of the problems inherent in the C-alkylation sequence. Dione 52 can be derived from enone 54 by a series of reactions analogous to that employed for the preparation of dione 53.



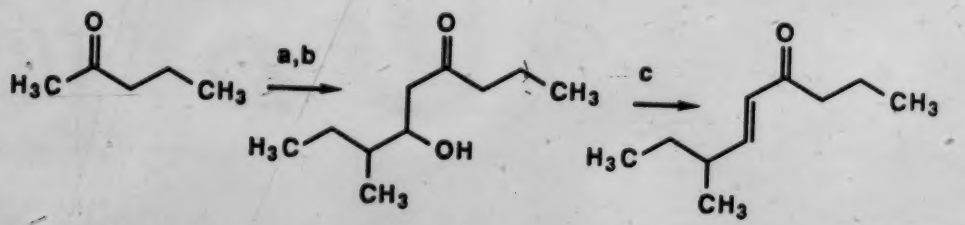
52 54

We considered the use of an aldol reaction for the formation of enone 54. However, we anticipated that an acid or base catalysed aldol may yield the undesired enone 74.[51]



56 48 74

For this reason, the *directed* aldol reaction was employed.[62] This method of preparation of 54 was preferred over the "Wittig method" since the required Wittig reagent would not be readily available. Thus treatment of ketone 56 with one equivalent of lithium diisopropylamide (LDA) (tetrahydrofuran (THF), -78°), followed by addition of aldehyde 48, afforded, after quenching with saturated NH_4Cl, hydroxy ketone 75. The crude alcohol was dehydrated with *p*-toluenesulphonic acid in benzene (24h) in the presence of molecular sieves to yield the desired enone 54 in 85-90% yield. This method of dehydration was chosen over the more classical Dean-Stark trap procedure because it afforded a 'cleaner' enone.



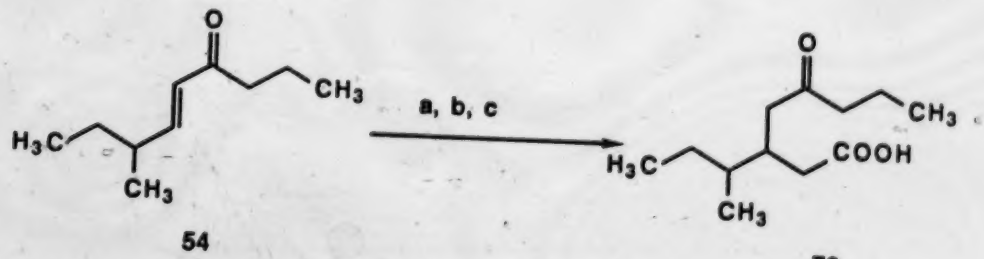
56 75 54

a) LDA, THF b) 48 c) pTSOH / PhH / Molecular Sieves

The ^1H nmr spectrum of 54 displays two *trans* olefinic protons (δ6.07 and 6.74, dd, J = 16.0 Hz) and a methylene triplet (δ2.51).

With enone 54 in hand, we applied the reaction conditions used for the formation of 58. Condensation of

enone 54 with diethyl malonate and NaOEt in refluxing dry ethanol for two hours, followed by base hydrolysis, afforded a major product whose R_f (tlc) and spectral data did not correspond to those expected for dione 52. The ir spectrum of this product shows absorption bands characteristic of a carboxylic acid (3400-2400 cm^{-1}). The hreims gives a formula of $C_{12}H_{22}O_3$ for the molecular ion. The ^1H nmr spectrum displays no olefinic signals. The spectral data are consistent with structure 76 for this product.

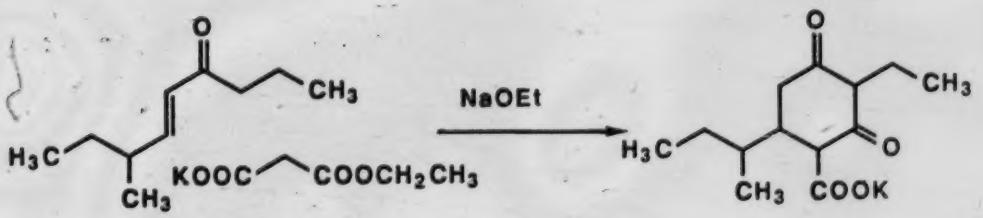


54 76

a) $CH_2(COOCH_2CH_3)_2$, NaOEt, EtOH
b) KOH, H_2O c) HCl, H_2O

We considered that keto acid 76 may arise by basic hydrolysis of intermediate 78 (see scheme 15). In order to eliminate the hydrolysis step, we envisioned using an alternate condensation agent. One such reagent replaces a ester group of diethyl malonate with a carboxylic acid salt. Addition of reagent 82 to enone 54 should give intermediate 83 which should readily decarboxylate, upon protonation, to afford 52. Thus, a mixture of monoethyl

potassium malonate (82)[63] and enone 54 was refluxed in absolute ethanol for 24 hours to effect condensation. Upon acidification of the hot reaction mixture, decarboxylation occured spontaneously, giving dione 52 in 65% yield (based on recovered starting material). The actual yield is, however, substantially lower (26%) owing to the poor solubility of monoethyl potassium malonate in ethanol.

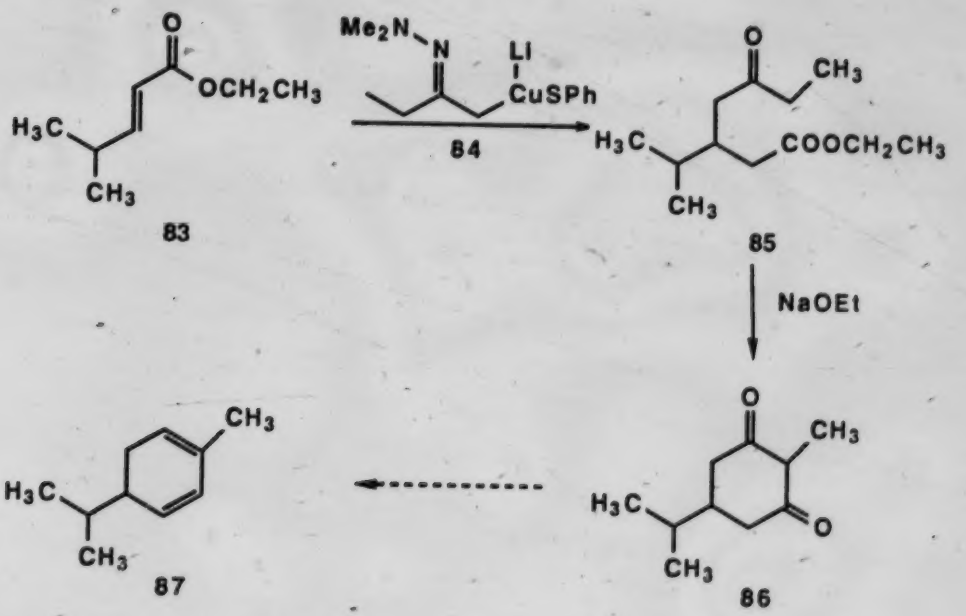


54 82 83

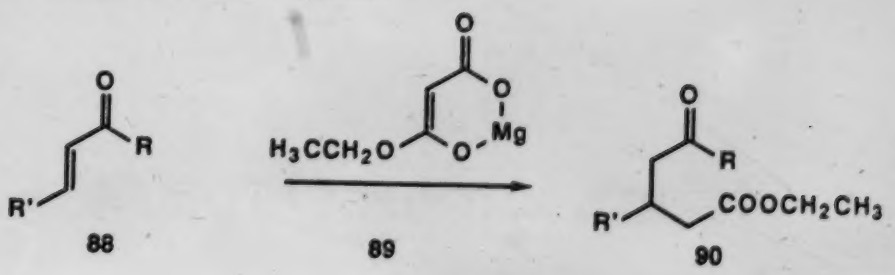
We investigated the stepwise formation of 52, i.e., Michael addition followed by Dieckman condensation. A similar sequence had been used by Paquette and Doehner in a synthesis of phellandrene (87) and is outlined in scheme 13.[64] They utilized a method involving metallation of the ketone N,N-dimethylhydrazone and conversion into its cuprate form 84 by reaction with cuprous thiophenoxide. A much simpler method for the preparation of analogs of 85 involves the use of magnesium monoethyl malonate (89) as the Michael donor. This reagent was developed by McMurry *et al* for the introduction of an acetate synthon to the

β-position of a mono-β-substituted enone (88) to give a keto ester (90) as outlined in scheme 14.[65]

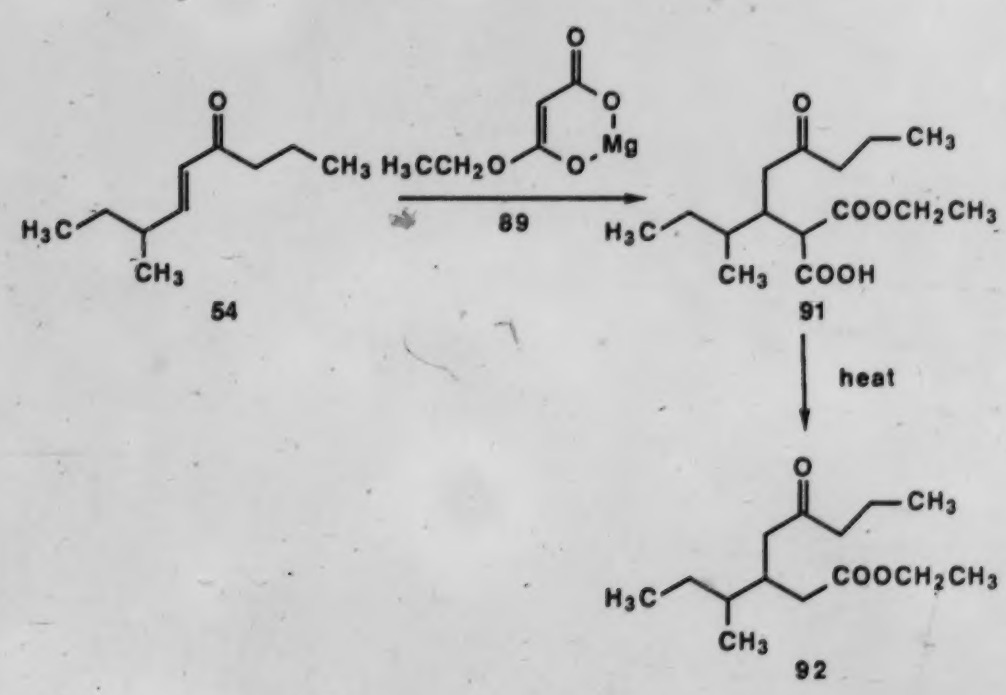
Scheme 13 : Paquette's synthesis of phellandrene (87)



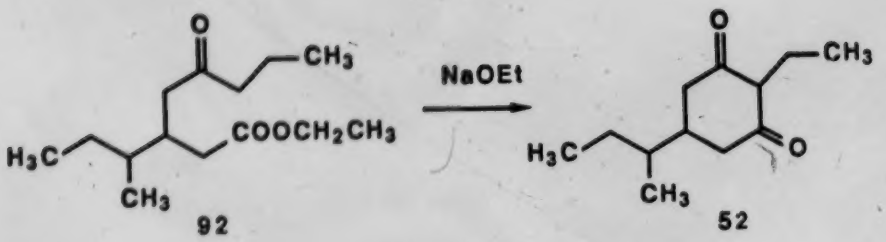
Scheme 14 : McMurry's acetate synthon



We decided to apply McMurry's method to enone 54. Thus, treatment of 54 with magnesium monoethyl malonate (89)[66] in dry dimethylformamide, followed by acidification afforded carboxylic acid 91. After heating to effect decarboxylation, keto ester 92 was obtained in very good yield. Its ir spectrum shows the absorptions for the ester and ketone carbonyls at 1734 and 1715 cm^{-1} respectively. The ^1H nmr spectrum of 92 displays an isolated ethoxyl group (δ4.11, q, coupled to δ1.24, t).



Subsequent Dieckman condensation of 92 using sodium ethoxide in toluene / ethanol afforded the desired dione 52 in 64% yield.



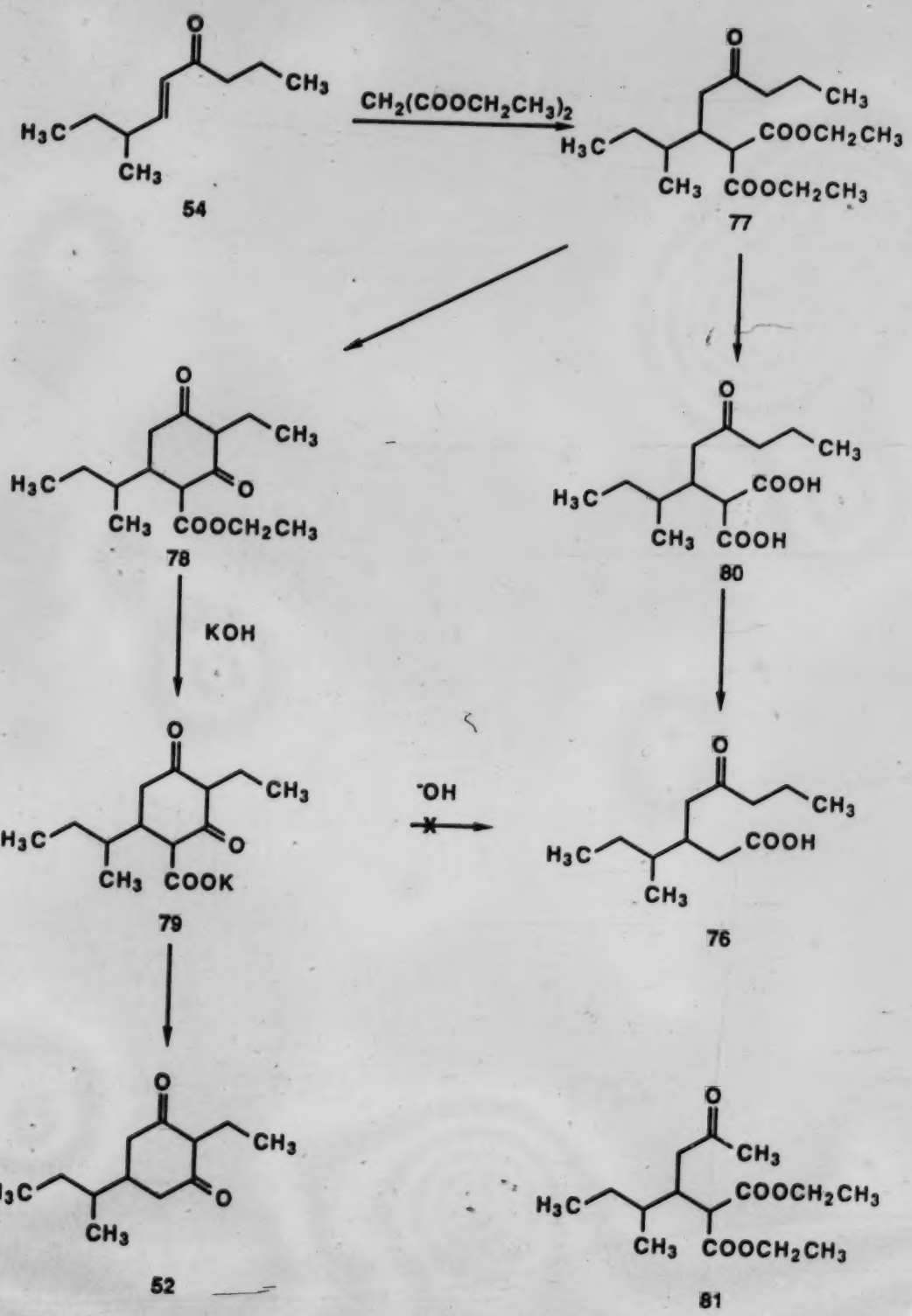
92 52

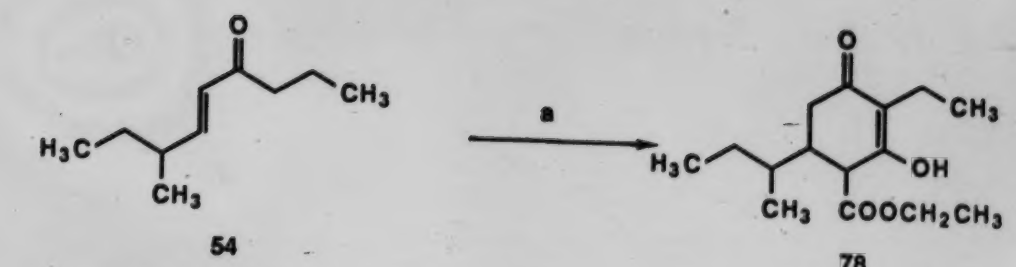
Since compound 92 could be converted to dione 52 under basic conditions, we conclude that compound 76 may arise by the hydrolysis of 77 and subsequent decarboxylation of intermediate 80 as shown in scheme 15. If such is indeed the case, the formation of 76 will be avoided by increasing the reaction time for the Dieckman condensation of 77. The slower relative rate of the Dieckman reaction for the propyl ketone 77 *versus* the methyl ketone 81 is reasonable due to the added steric bulk in the former case.

The condensation of enone 54 and diethyl malonate for 12 hours gave keto ester 78 in 80-92% yield. The ^{1}H nmr spectrum of 78 shows it exists as the enol tautomer. It displays quartets for the methylene protons of the ethoxyl and the C-2 ethyl group (δ4.25 and 2.31 respectively) as well as an enolic hydroxyl proton (broad singlet, δ8.9).

Treatment of dione ester 78 with aqueous potassium hydroxide under reflux for 12 hours, followed by acid workup, gave dione 52 in excellent yield.

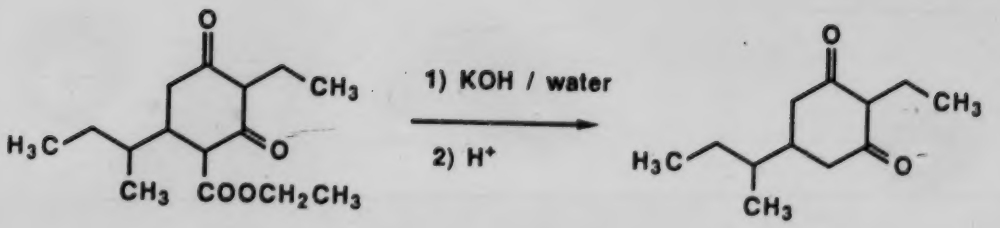
Scheme 15 : Formation of 76 and 52 from 54





54 78

a) $CH_2(COOCH_2CH_3)_2$, NaOEt, EtOH



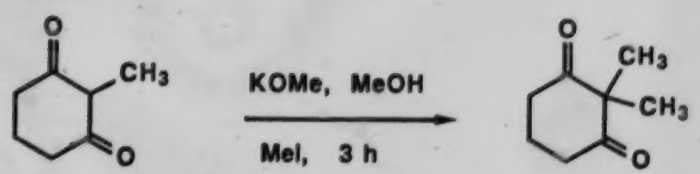
78 52

In summary, the preparation of dione 52 by condensation of diethyl malonate with enone 54, followed by hydrolysis and decarboxylation is the most efficient method.

It is now necessary to introduce the quaternary methyl group *via* an alkylation reaction. In addition to the inherent difficulties of the C-alkylation of a cyclic β-diketone (*vide infra*), there is another problem: a 2,2-disubstituted- 1,3-cyclohexanedione, is very sentitive

to nucleophilic ring cleavage.[67] Hence relatively hindered bases must be used for the alkylation to prevent this side reaction.

Introduction of a methyl group is a reasonably facile C-alkylation process. Good yields of 2,2-dialkylated dione can be obtained even with nonhindered bases such as potassium methoxide.[68] This can be accomplished without significant ring cleavage. When the C-2 substituent is not a methyl group, however, the rate of alkylation decreases substantially and side reactions predominate.[69] For this reason, bulkier bases, such as potassium *tert*-butoxide, are generally used.[70]

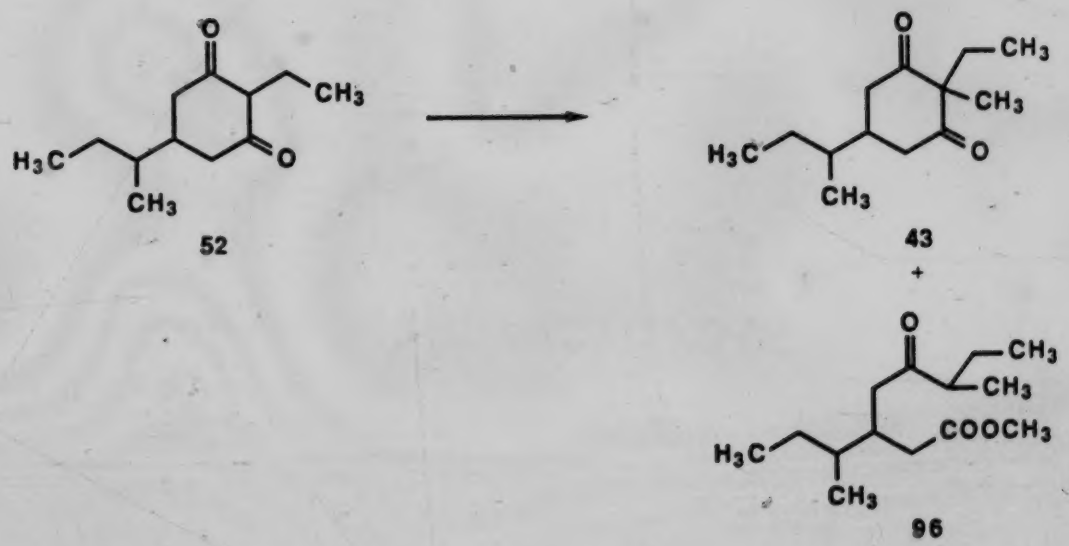


65 93

As a model system for the second alkylation step of 52, we looked at the formation of 2,2-dimethyl-1,3-cyclohexanedione (93) from the 2-methyl cyclohexanedione (65). Literature reports indicate that the best conditions for the preparation of 65 make use of potassium tert-butoxide in tert-butyl alcohol with

iodomethane as the alkylating reagent.

When dione 52 was treated with iodomethane and potassium *tert*-butoxide in anhydrous *tert*-butyl alcohol, three products were obtained. The desired dione 43 was obtained in low yield (30-40%). Its ir spectrum shows absorptions at 1721 and 1694 cm^{-1} for the diketone. The ^1H nmr spectrum of 43 shows a singlet at δ1.18 (major isomer) and one at δ1.15 (minor isomer), corresponding to the quaternary methyl group of the two sets of diastereomers where the methyl group is *cis* and *trans* to the *sec*-butyl side chain. The undesired O-alkylated product 95 was also formed, but in relatively low yield.



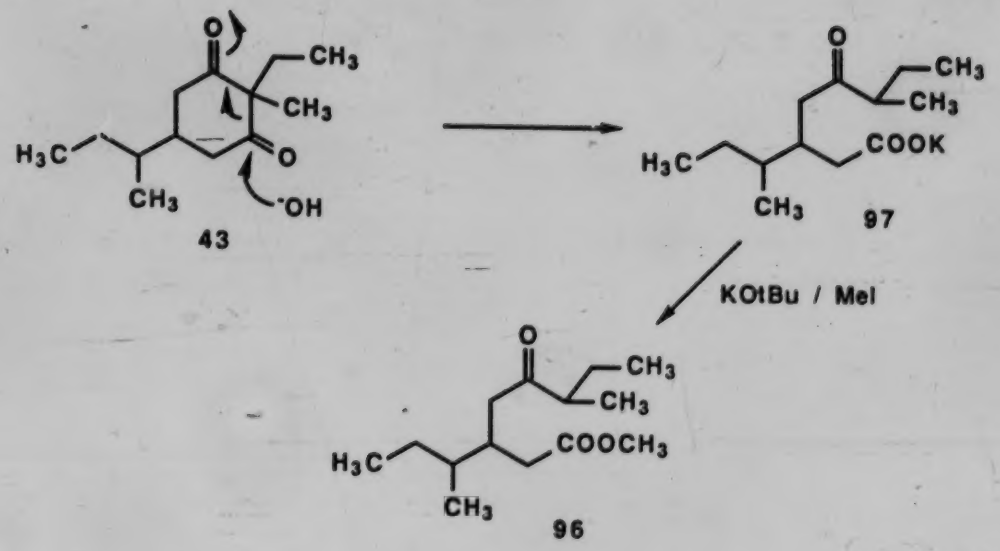
In addition to the C- and O-alkylated products 43 and 95, a third product was isolated in variable yields (20-50%). Its ir spectrum shows absorptions at 1733 and 1712 cm^{-1} characteristic of ester and ketone carbonyls respectively. The ^1H nmr spectrum shows a singlet a δ3.65 corresponding to a methoxyl group but it does not show a methyl singlet. Structure 96 was assigned to this product in accordance with the spectral data obtained. This assignment was confirmed later by comparison of its spectral characteristics with those of another keto ester (98) prepared by a different route (*vide supra*).

The presence of keto ester 96 can be explained as illustrated in scheme 16. As dione 43 is generated, the α,α-disubstituted-β-diketone, being very susceptible to nucleophilic ring cleavage by hydroxide ion, reacts to produce keto acid 97. The resulting acid can then be methylated, under the reaction conditions, to yield keto ester 96.

When precautions were taken to guarantee the rigorous exclusion of any hydroxide, i.e., redistilling the *tert*-butyl alcohol from potassium under argon and generating the potassium *tert*-butoxide *in situ* from potassium metal, much better yields of dione 43 (60-65%) were obtained. Keto ester 96 was produced in very small amounts (less than 10%).

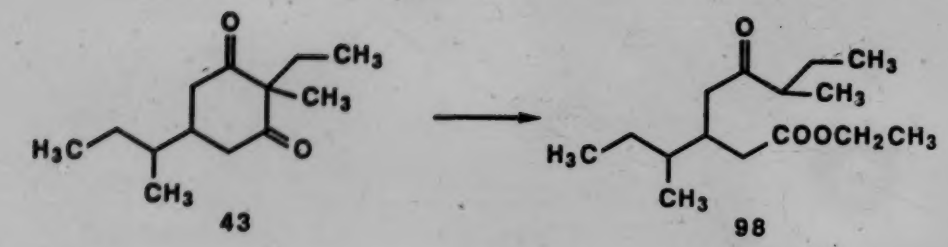
With dione 43 in hand, it only remains to introduce the formyl group and the double bond. A preliminary

Scheme 16 : Formation of 96 from 43

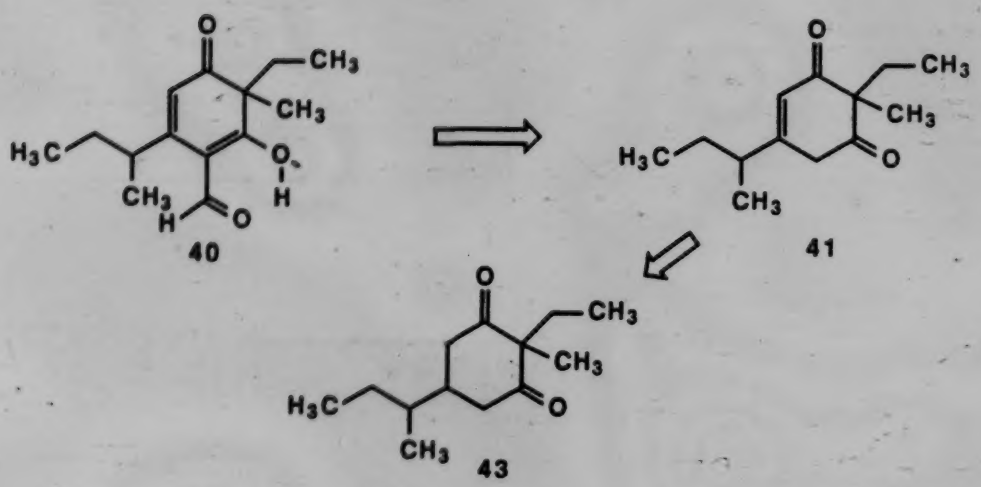


attempt at introducing the formyl group using a standard method (NaH / HCOOEt / THF [EtOH])[71] produced keto ester 98 as the major product. Dione 43 is very sensitive to nucleophilic ring cleavage thus formylation should be preferably carried out after the introduction of the C-4 C-5 unsaturation. The acidity of the C-6 protons in keto enone 41 should protect the molecule against nucleophilic cleavage.

Several methods are available for the conversion of saturated ketones to α,β-unsaturated ketones. One of the most common one involves the halogenation / dehydrohalogenation sequence (scheme 17).[72] An alternative



43 98

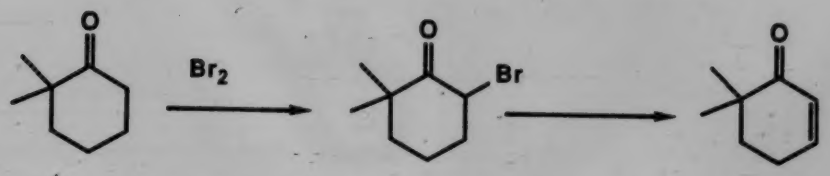


40 41 43

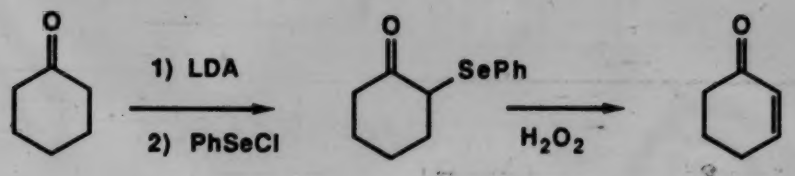
method involves the formation of the α-seleno ketone followed by oxidation and elimination of the resulting selenoxide (scheme 18).[73] For the preparation of 41 we

require a mild conversion method, i.e., one that does not involve nucleophilic reagents, and one which will allow for a monofunctionalization of the symmetrical diketone. One existing method (scheme 19), the oxidation of trimethylsilyl enol ethers to enones with dichlorodicyanobenzoquinone (DDQ)[74] or palladium acetate - *p*-benzoquinone,[75] is in fact, very well suited to our purposes. Indeed, formation of a bissilyl enol ether 105 allows for only one available site for oxidation by DDQ.

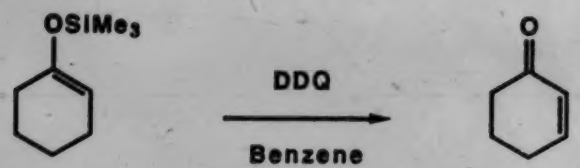
Scheme 17 : Bromination - dehydrobromination sequence

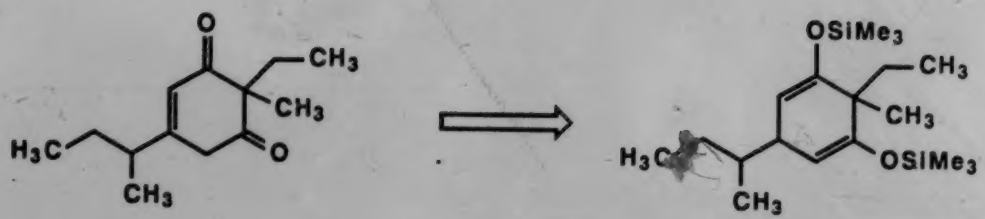


Scheme 18 : Selenoxide route



Scheme 19 : DDQ dehydrogenation

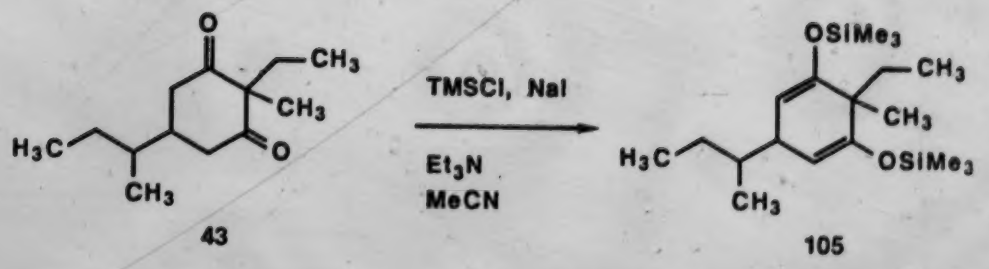




41 105

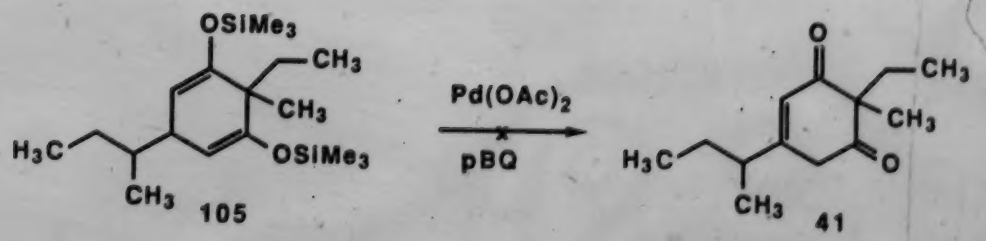
Treatment of dione 43 with chlorotrimethylsilane and sodium iodide in acetonitrile for 30 min at room temperature afforded the bissilyl enol ether 105 in very good yield.[76] Its ^1H nmr spectrum displays a doublet at δ4.62 (J = 3 Hz) corresponding to the olefinic protons, a singlet at δ0.18 for the trimethylsilyl methyl groups, and signals attributed to *sec*-butyl, ethyl and methyl groups.

Treatment of 105 with palladium acetate and *p*-benzoquinone did not afford any trace of oxidized



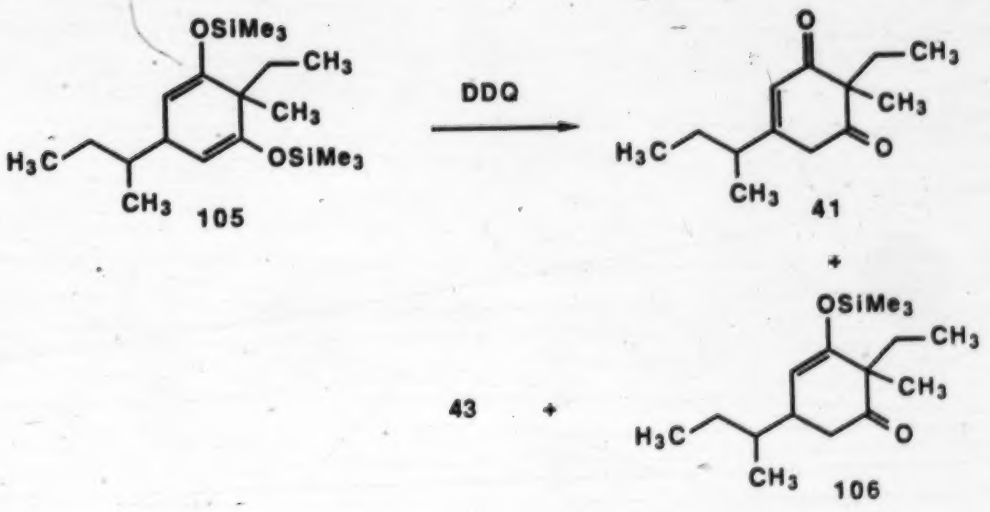
43 105

product. However, treatment of 105 with DDQ in benzene gave the desired keto enone 41 in low yield (21%), along with other products.



105 41

The ir spectrum of keto enone 41 shows absorptions at 1726 and 1665 cm^{-1} corresponding to the saturated ketone and the α,β-unsaturated ketone respectively. The 400 MHz ^1H nmr spectrum shows signals expected for the alkyl substituents, a vinyl proton (δ6.08), and the C-6 methylene protons as 2 sets of AB systems centered at δ3.20



corresponding to the two diastereomers present (1:1 ratio).

The mono-silylated ketone 106 was isolated in 13% yield. Its ^{1}H nmr spectrum shows the olefinic protons at δ4.90 along with the trimethylsilyl group at δ0.26. The ir spectrum of 106 shows absorptions at 1715 cm^{-1} (ketone carbonyl), and 1655 cm^{-1} (enolic double bond).

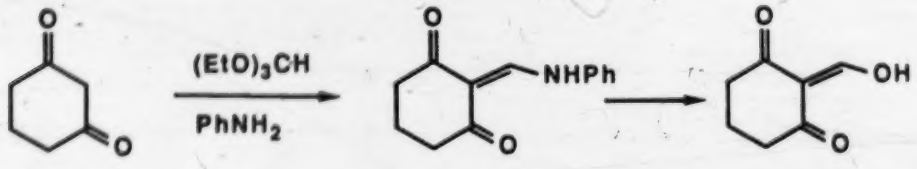
The final step in the synthesis of ceratenolone requires a formylation reaction. Acylation at carbon under basic conditions using ethyl formate is an efficient process.[77] The experimental conditions usually involve either sodium hydride in ether, or sodium methoxide in benzene.[78]

When enone 41 was treated with sodium hydride and ethyl formate in tetrahydrofuran at room temperature, no reaction took place; only starting material was recovered. No reaction was observed when the reaction was carried out in refluxing THF for 48 hours, or in refluxing ethyl formate for 24 hours. It was thought that the temperature obtained in these two solvents was not high enough to provide the activation energy needed for the reaction (THF bp: 67°, EtOCHO bp: 52°). In an attempt to overcome this problem, forcing conditions were employed. The reaction was carried out in a sealed tube, using ethyl formate as the solvent, in a steam bath. After 36 hours reaction time, no trace of ceratenolone could be detected (by tlc or by nmr of the residue after removal of ethyl formate). It thus appears that the enolate of 41 is very stable, presumably by virtue of its extended delocalization and, as a consequence, is very unreactive.

41 40

Since the enolate of 41 is stable, it should be possible to introduce the formyl group using methods developed to formylate other stable enolate systems. Indeed, active methylene compounds, such as cyclic β-diketones, can be acylated under acidic conditions.[79] One method uses triethyl orthoformate and aniline to generate the enamine.[80] The enamine is readily hydrolysed to the free formyl group, as shown in scheme 20.

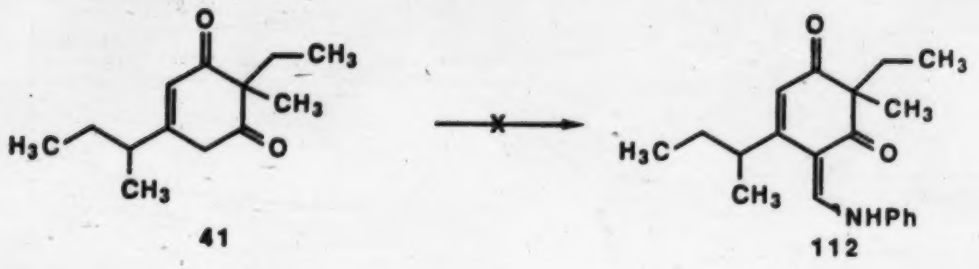
Scheme 20 : Acylation under acidic conditions



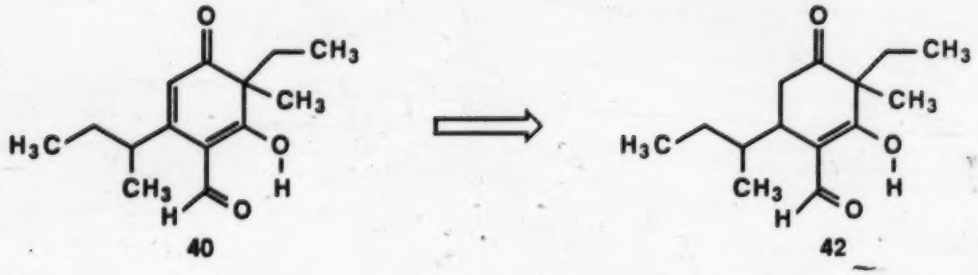
109 110 111

When keto enone 41 was treated with triethyl orthoformate and aniline in acetic acid, a very complex mixture of products was obtained. Variations of the procedure were investigated, including the use of neat triethyl orthoformate, but in no case was the desired formyl group introduced.

The difficulties encountered in the formylation of 41 prompted us to consider an alternative sequence; introduction of the formyl group, followed by dehydrogenation. In principle, the double bond should be



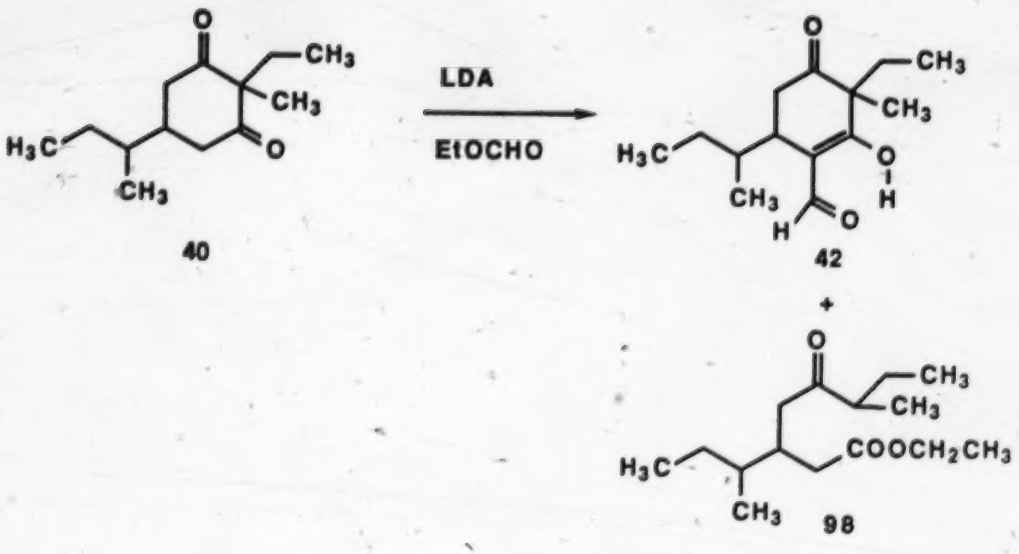
readily introduced by oxidation with DDQ[78,81] or other dehydrogenating agents.



The problem now becomes that of introducing a single formyl group on diketone 43, while preventing ring opening as a side reaction. The standard methods of formylation are not applicable with this substrate due to the sensitivity of 43 to nucleophiles, as mentioned earlier. We decided to form the monoanion of 43 under selective conditions and attempt to trap this anion with ethyl

formate. Diketone 43 was treated with LDA at -78° in tetrahydrofuran for 60 min, followed by addition of ethyl formate at -78°. After one hour at -78° there was no sign of reaction (tlc), and the solution was warmed to 0°. After 8 hours at 0°, there was still no sign of reaction and the solution was then allowed to warm to room temperature overnight. Tlc of the reaction mixtute revealed that the desired formate had formed as evidenced by the purple color obtained for its spot when the tlc plate is dipped into a ferric chloride solution. After workup of the reaction, however, it was found that the the yield of sodium hydroxide soluble products was less than desirable (11%). A longer reaction time (30 hours) increased the reaction yield to 24%. The ^{1}H nmr spectrum of the crude acidic extract showed that it consisted almost exclusively of the desired keto formate 42. The ^{1}H nmr spectrum of 42 shows signals characteristic of the alkyl substituents as well as two low field signals at δ8.71 and 15.07 corresponding to the enolized formyl group hydrogen and hydrogen-bonded hydroxyl proton, respectively. This indicates that the tautomer present is that shown in 42.[82]

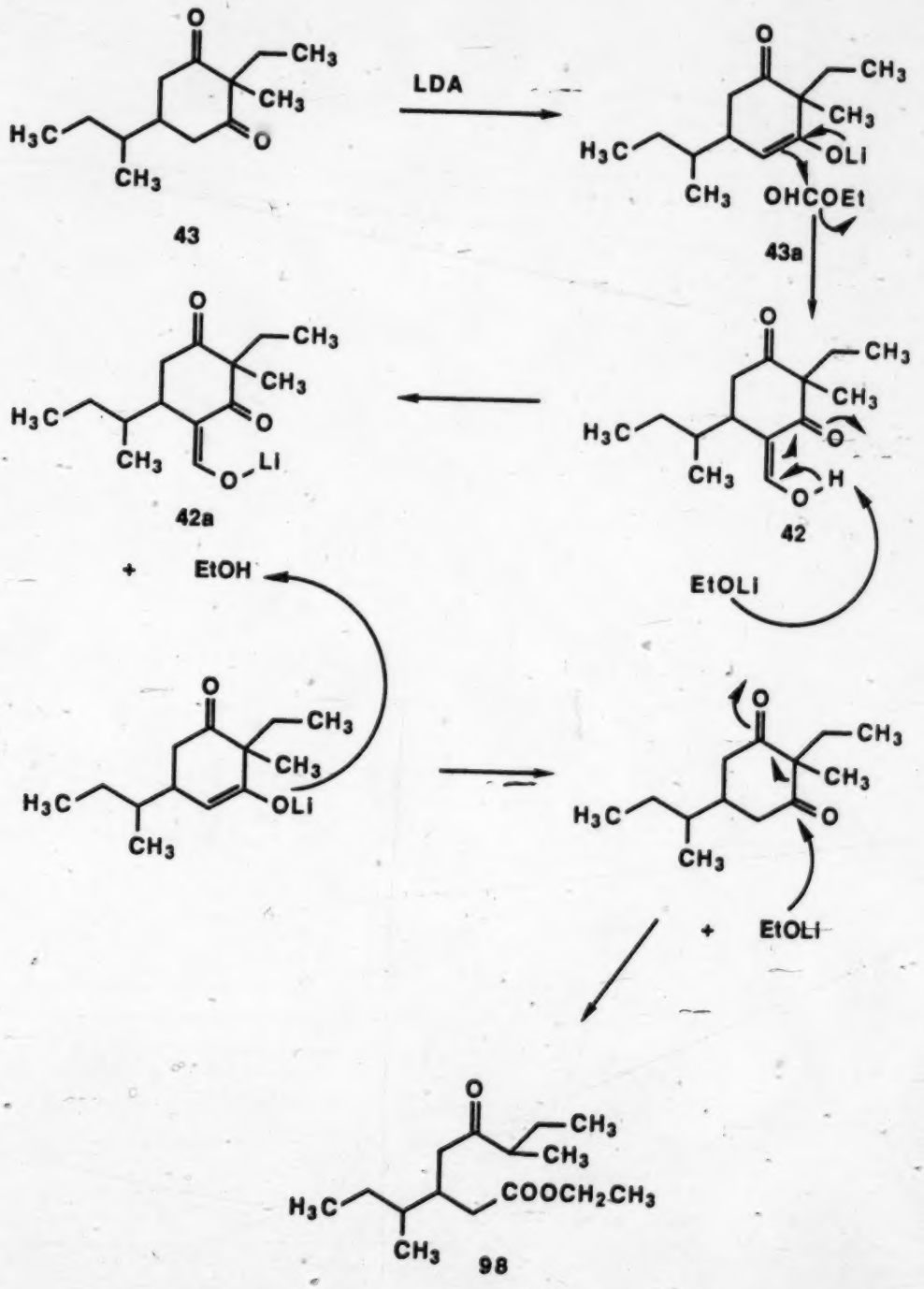
The fraction that was not soluble in aq. sodium hydroxide accounted for the remainder of the dione used. Surprisingly, however, a product other than recovered starting material was present (*ca* 1:2 ratio, ^{1}H nmr). It was found to be keto ester *98*. The ^{1}H nmr spectrum of 98 shows a quartet at δ4.11 for the ethoxyl group as well as



signals characteristic of the alkyl groups.

The formation of 98 may be explained according to scheme 21. Enolate 43a reacts with ethyl formate generating ethoxide ion. The ethoxide ion abstracts the acidic α-proton of compound 42, producing anion 42a and a molecule of ethanol. Ethanol should react in turn with another molecule of enolate 43a, regenerating ethoxide ion and dione 43. In the presence of ethoxide ion, dione 43 undergoes nucleophilic ring cleavage to produce compound 98. Therefore according to the stoichiometry implied by scheme 21, the maximum yield of 42 attainable for this reaction would be 50%.

Scheme 21 : Formation of 98 from 43

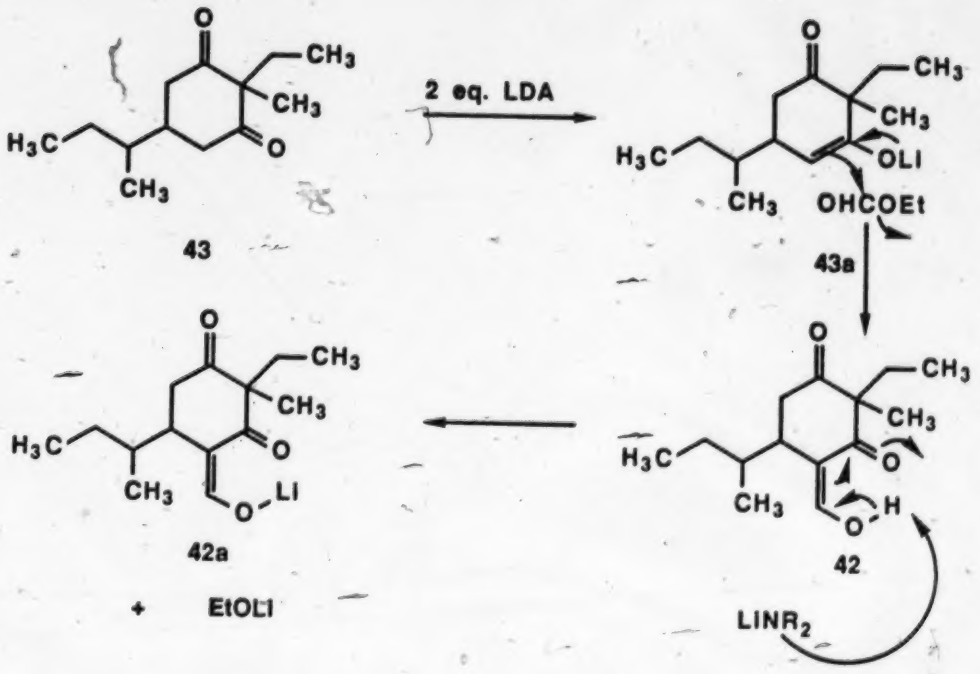


Further conclusions can be drawn from these observations. The formylation reaction is not kinetically controlled. It is, in fact, quite slow even at room temperature. This is in contrast to the formylation of other ketones which proceed quite readily at or below room temperature with ethyl formate as the formylating agent.[77] The slow reaction rate may further explain why the formylation did not proceed at all with enone 41. Since the anion of 41 is conjugated, it is expected to be less reactive than the anion of dione 43.

The slow formylation of dione 43 can be explained by steric hindrance. Examination of molecular models for the various conformations of the enolate of 43 reveals that there is moderate steric hindrance to approach to the α position of the ketone. This is the result of a *pseudo* 1,3-diaxial interaction with the axial methyl (or ethyl) group at C-2 on one side. Approach of the reagent from the other side is slightly hindered by the relatively bulky *sec*-butyl group.

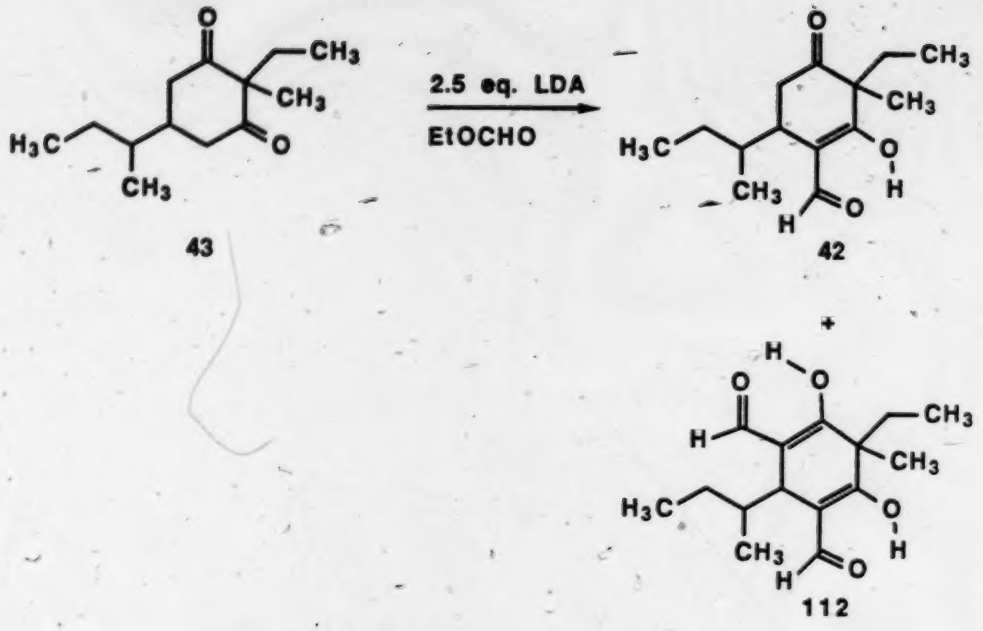
One way to overcome ethanol quenching of the enolate and the ensuing ring opening would be to use two equivalents of LDA. As shown in scheme 22, the excess base would probably deprotonate the α-formyl ketone produced faster than would ethoxide ion, thereby preventing the formation of ethanol. Formation of a diformylated product is possible when using two equivalents of base.

Scheme 22 : Proposed formation of 98 from 43



When dione 43 was treated with 2.5 equivalents of LDA and 1.0 equivalent of ethyl formate for 40 hours at room temperature, the desired mono-formylated product 42 was isolated in 61% yield. However, the reaction mixture contained another hydroxide soluble product in addition to compound 42. Its spectral data corresponds to that of the diformylated product 112. The neutral portion of the reaction mixture contained mainly unreacted dione 43 and some ring opened keto-ester 98.

With the α-formyl ketone 42 in hand, we needed a method of effecting the desired dehydrogenation. In addition to the dehydrogenation methods mentioned earlier

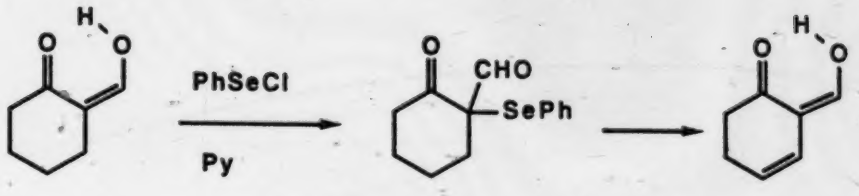


43 42 112

to prepare α,β-unsaturated ketones, there are methods specific to the dehydrogenation of α-formyl ketones. The more classical approach is the direct dehydrogenation using quinones, as illustrated in scheme 24.[81,83] Reich *et al* have selenated β-dicarbonyl compounds by first reacting these compounds with sodium hydride in THF, followed by reaction of the resulting anion with phenylselenyl chloride. Oxidative selenide elimination then affords the desired unsaturated β-dicarbonyl compound.[84] Liotta *et al* have reported a simpler and more efficient variation of the method. The dicarbonyl compound is reacted with a 1:1 pyridine / phenylselenide complex in dichloromethane, to

afford the intermediate selenide in excellent yield (scheme 23).[85]

Scheme 23 : Dehydrogenation via the selenoxide



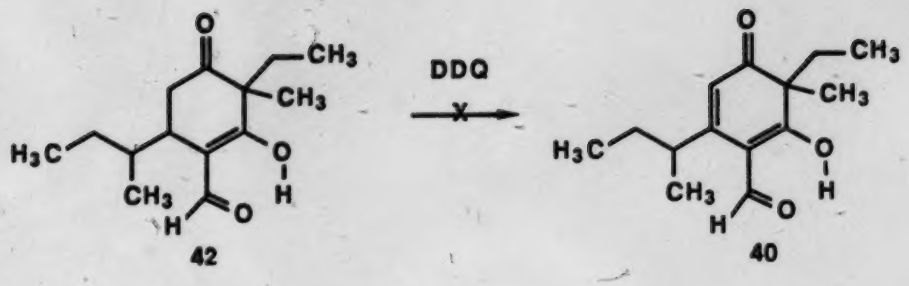
113 114 115

Scheme 24 : Dehydrogenation with DDQ

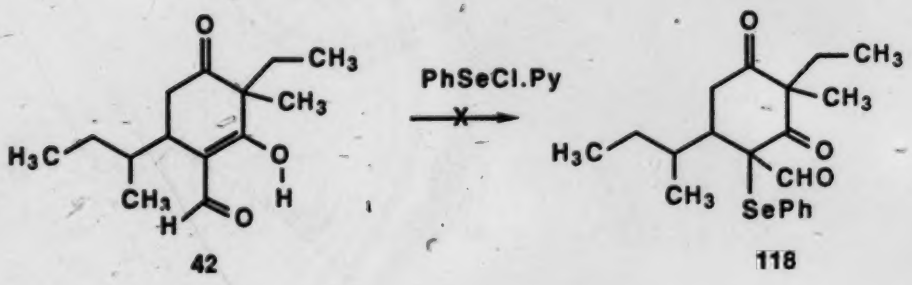
116 117

When 42 was treated with DDQ in benzene or dioxane at room temperature for 24 hours no reaction was observed. Even when 42 and DDQ were heated in refluxing toluene, the starting material was recovered.

Attempts to effect dehydrogenation of 42 *via* introduction of an α-phenylselenide (and subsequent oxidative elimination), were thwarted. We were unable to

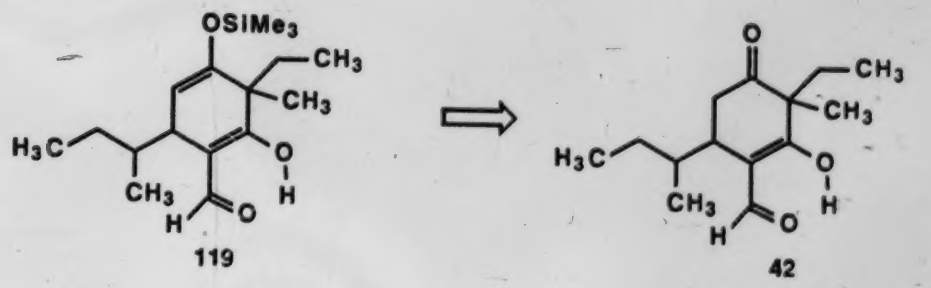


form the α-formylselenide upon treatment of 42 with the phenylselenyl chloride - pyridine complex. This was unexpected since, in general, this reaction is reported to be over within 15 minutes.



However, the inability to effect dehydrogenation can be accounted for by the steric bulk of the reacting species, together with the sterically hindered nature of the α-position of the ketone. In order to circumvent this problem, we proposed to activate the molecule towards

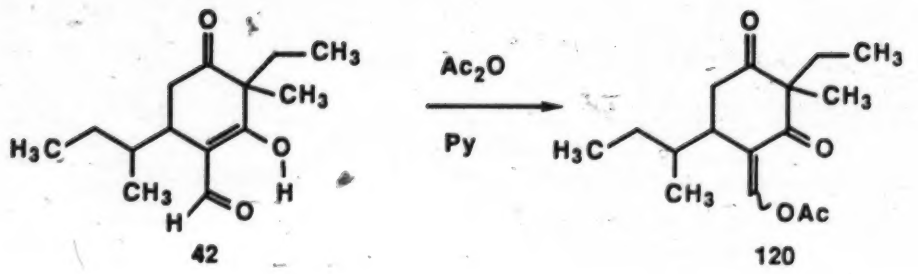
oxidation by preparation of its silyl enol ether 119. This should serve two purposes; it creates a second site of attack available to DDQ and it modifies the conformation of the molecule.



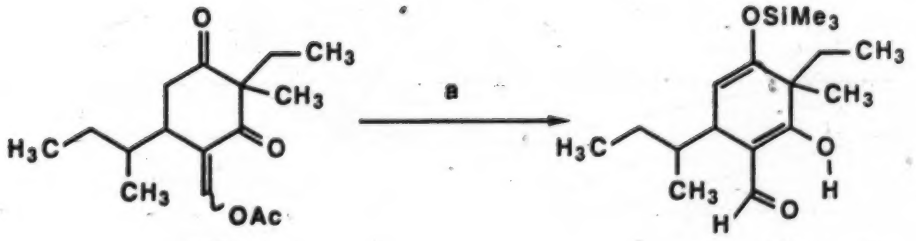
Attempted silylation of 42 resulted in recovery of the starting material. Since the formyl group may be interfering with the silylation reaction we decided to mask it by forming the corresponding acetyl derivative. Thus treatment of 42 with pyridine - acetic anhydride afforded the diketo acetate 120.

Treatment of compound 120 with chlorotrimethylsilane, sodium iodide and triethylamine in acetonitrile[76] afforded in very good yield the desired silyl enol ether 119 in which the acetyl group had been cleaved, and a small amount of 121.

It is surprising that 120 produces good yields of product under the silylating conditions while 42 is unreactive. This may be explained by the fact that the

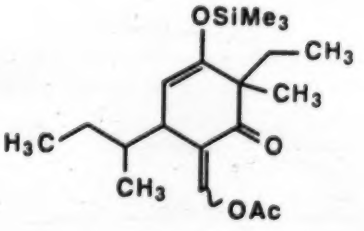


42 120



120 119

a) TMSCl, NaI, Et_3N, MeCN



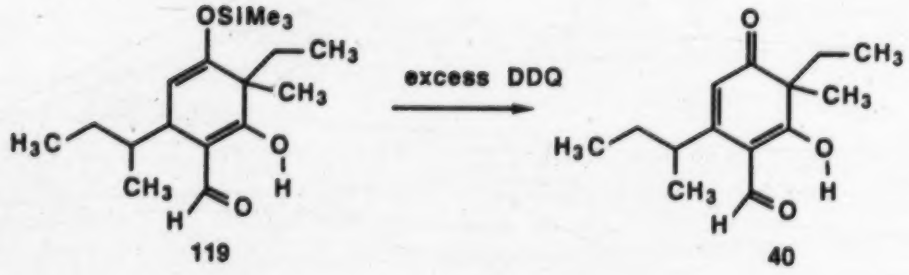
121

major tautomer of an α-formylcyclohexanone is the aldo-enol form and not the hydroxymethylene-keto form as is often assumed. On acetylation, α-formylketones are reported to

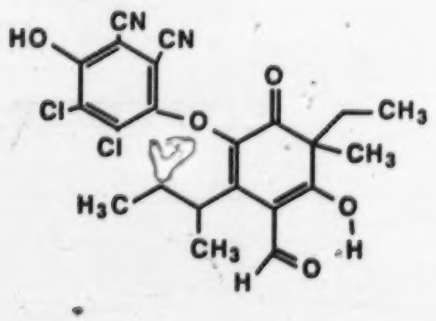
form the E-*exo*-acetoxymethylene compounds.[82] The spectral data reported for such compounds agree very well with those of 120. The ^{1}H nmr spectrum of 120 shows the olefinic signal at δ8.01 and 8.06. We must, therefore, conclude that 120 exists mainly as the E-*exo*-isomer. It is not clear how the two different conformations, the aldo-enol form for 42 *vs* the hydroxymethylene - keto form for 120, affect the enolization of the other ketone. The *bis*-silylation of 43 proceeds smoothly *via* the monosilylated compound 106, which should adopt a conformation very similar to that of the aldo-enol tautomer of 42.

When 119 was subjected to the action of DDQ in benzene - chloroform (1:1), the starting material was slowly consumed. Analysis of the chloroform solution by tlc showed a spot giving the characteristic violet color of ceratenolone upon exposure to ferric chloride. The ^{1}H nmr spectrum of the solution confirmed that ceratenolone had formed, as it contained the same low field signals as those of the natural product. Purification of the crude reaction product by flash chromatography afforded ceratenolone (1). The ir, hreims, and 200 MHz ^{1}H nmr spectra of 1 are indistinguishable from those of the natural product. The 400 MHz ^{1}H nmr spectrum shows doubled signals for the C-8, C-12, and C-13 methyl groups. This is in agreement with a 1:1 mixture of diastereomers being present.

Another product, 122, is formed in this reaction. Its formation becomes more important as the scale of the reaction is increased. It gives a green color upon exposure to ferric chloride. The ^1H nmr spectrum of 122 displays signals characteristic of the alkyl groups of ceratenolone, but no olefinic proton. Its hreims shows a peak corresponding to an ion of formula $C_{22}H_{20}O_5Cl_2N_2$. The spectral data is consitent with an adduct of ceratenolone and DDQ, and structure 122 was assigned to this product. When ceratenolone was treated with DDQ, it was completely converted to the adduct 122, although relatively slowly.

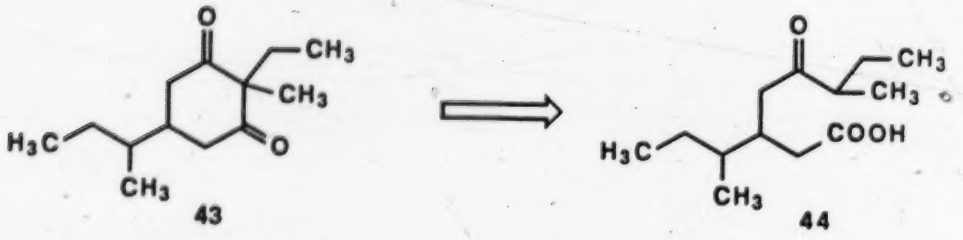


119 40



122

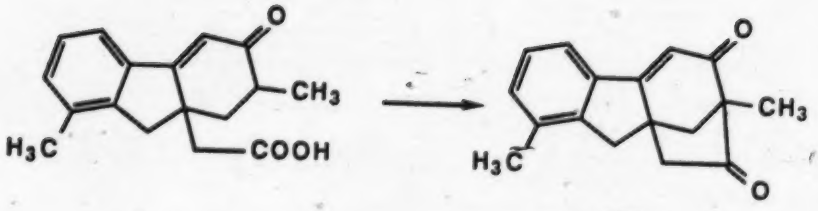
As mentioned earlier, we considered several routes for the preparation of intermediate dione 43. The synthesis 43 employing an "acyclic" route (see scheme 8) potentially offers the advantage of avoiding the alkylation step by incorporating both the ethyl and methyl group substituents into the starting enone. Keto acid 44 is an attractive intermediate as it should be possible to effect cyclisation to 43 by intramolecular acylation and 44 should be readily available from enone 45. A similar intramolecular acylation reaction has precedent in the literature in the synthesis of 123.[86]



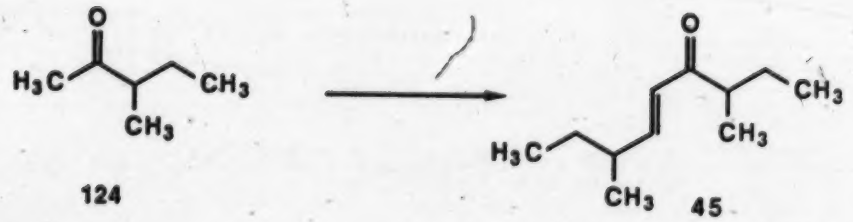
Enone 45 was prepared by treatment of the enolate of ketone 124, with aldehyde 48. Subsequent dehydration gave enone 45.

Michael addition of monoethyl magnesium malonate (89) to enone 45 in DMF, followed by heating of the resulting acid 125 to ca. 160° to effect decarboxylation, afforded keto ester 98 (Scheme 25). Hydrolysis (KOH, EtOH) afforded

44 45



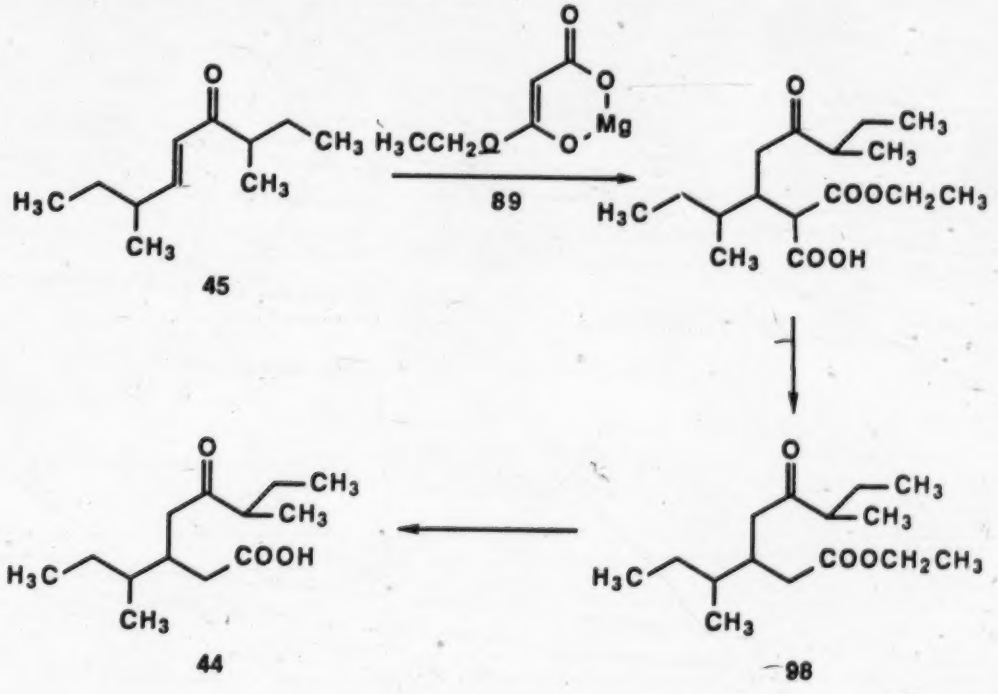
123



124 45

keto acid **44** in 31% overall yield. The ir spectrum of **44** shows a broad absorption at 2900 and 1735 cm^{-1} corresponding to the carboxylic acid and a strong absorption at 1708 cm^{-1} characteristic of a ketone carbonyl.

Scheme 25 : Formation of 44 from 45



When ketoacid **44** was treated with boron trifluoride and acetic anhydride in acetic acid,[86,87] no cyclized product was detected amongst a complex mixture of products (tlc analysis and the absence of methyl singlets (at δ1.18) in the ^1H nmr spectrum of the reaction product). The desired dehydration was achieved by heating ketoacid **44** in toluene in the presence of *p*-toluenesulphonic acid, using a

Dean-Stark apparatus.[88] The formation of 43 was evident by tlc and by nmr of the crude reaction product (methyl singlet at δ1.18). Purification by flash chromatography afforded pure 43 in low yield (38%, non-optimized).

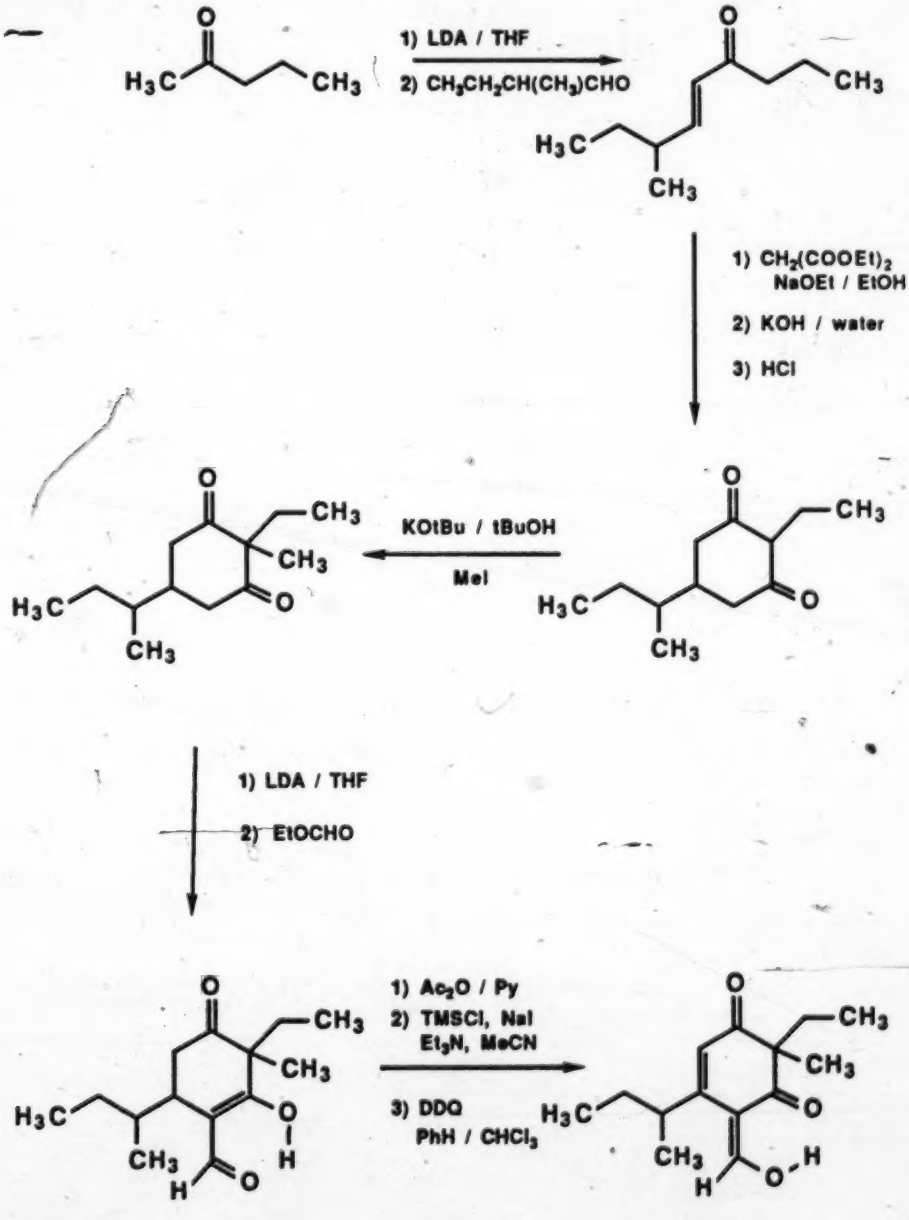
The successful synthesis of ceratenolone and epiceratenolone is summarized in scheme 26. The problem encountered in the last step, i.e., the formation of an adduct with DDQ, could possibly be solved by using a less reactive quinone but this has not yet been investigated. Other means of dehydrogenating 41 should also be explored.

Ceratenolone prepared by this route consists of a 1:1 diastereomeric mixture. A single spot is obtained on tlc using several solvent systems. This is not so surprising considering that the two pairs of diastereomers differ only in the configuration of the *sec*-butyl side chain. This difference is not significant enough to give different signals for the various groups in the ^{1}H nmr spectrum at 200 MHz. However, formation of a suitable derivative might

allow for their separation.

However, we felt that the mixture of diastereomers of 1 is suitable to carry out the iron binding studies described in the next section.

Scheme 26 : Summary of the synthesis of ceratenolone



B. Iron binding studies

With ceratenolone (1) in hand, we undertook to study the complex formation between 1 and iron (III).

Theoretical background

Metal complexes can form between metal ions and organic molecules or ions.[89] The complexing agent with which a metal ion reacts is known as a ligand. Since ligands can be anions or neutral molecules, the complexes may be cations, neutral molecules or anions. For a substance to function as ligand, it must have at least one pair of electrons to donate a metal ion. The co-ordinating atoms of the ligand are usually oxygen, nitrogen, or sulfur. The maximum number of ligands that are bound by a metal ion is equal to its coordination number.

Metal ions in aqueous solution are themselves complexes because they orient the water molecules immediately surrounding them, leading, especially in the case of transition and higher-valent metal ions, to definite complexes such as $Fe(H_2O)_6^{3+}$. This is a consequence of the dipole moment of the water molecule, aligning with a specific spatial orientation to the ionic charge on the metal ion. For this reason, complex formation in solution is really a replacement process in which one or more of the solvent molecules surrounding the metal ion is replaced by other ions or molecules, to give complexes which usually have different chemical and

physical properties. Rates of exchange of water bound to metal ions are (for most ions) very much less than a millisecond ($t_{1/2}$).

In most cases, the formation and dissociation of complexes proceeds rapidly by a succession of equilibrium reactions. If M signifies a metal ion and L is a ligand, the following equilibria may be written.

$$M + L \rightleftharpoons ML$$

$$ML + L \rightleftharpoons ML_2$$

$$ML_{n-1} + L \rightleftharpoons ML_n$$

Because of these stepwise equilibria, a series of complexes may coexist in the solution.

The equilibrium reactions are described by a set of equilibrium ratios,

$$K_1 = [ML]/[M][L] \ , \ \ldots \ , \ K_n = [ML_n]/[ML_{n-1}][L]$$

which are known as formation constants, and the overall constant, β_n, is known as the overall stability constant of the complex ML_n.

$$\beta_n = [ML_n]/[M][L]^n$$

Extensive collections of formation and overall stability constants are available.[90]

Many factors influence the stability of complexes including the nature of the atoms concerned in bond formation and the base strength of the ligand. Increased stability occurs in complexes in which the ligand is attached to the metal by two or more atoms forming a chelate compound. If the metal is co-ordinated to the ligand by five- or six-membered rings, the complex shows a greatly increased stability and this effect is further enhanced if the ligand is multidentate (so that the complex contains two or more such rings). Ethylenediamine and the dianion of salicylic acid are examples of bidentate ligands. Qualitatively, the more points of attachment there are between a ligand and a metal ion, and the more basic the ligand, the more stable the complex.

A ligand is a basic molecule which may lose its complex forming properties when extensively protonated at low pH. Phenols for example, are weak acids (pKa 10) that form complexes *via* the phenolate ion. These compounds display a progressive decrease in their complexing properties at pH values less than 10. On the other hand, thiocyanate ion, derived from a strong acid, is almost pH-independent in complex formation ability.

Several methods have been developed to obtain the formation and stability constants of metal complexes and the topic has been extensively studied.[91-94] The experimental techniques developed include potentiometric, conductance, spectrophotometric, and polarographic methods.

In the potentiometric method, the problem is to determine the nature and concentrations of all the species present in the solution from the measured pH of solutions containing the metal ion, the ligand, and the acid (or base). In addition to the equilibrium ratio equations, mass balance and charge balance equations are also needed. For the complex formation reaction,

$$iM + jH + kL \rightleftharpoons M_iH_jL_k$$

the constants have the following form.

$$\beta_{MiHjKl} = [M_iH_jL_k]/[M]^i[H]^j[L]^k$$

The total concentration of the metal ion (T_M) may be written :

$$T_M = [M] + (\text{Sum of all species containing M} : i[M_iH_jL_k])$$

and the total ligand concentration (T_L):

$$T_L = [L] + (\text{Sum of all species containing L} : k[M_iH_jL_k])$$

A charge balance equation expresses the fact that the solution containing ions must be electrically neutral and, dropping the ionic charges for the sake of convenience, this can be expressed as

$$[H]+[B]+m[M]-[X]-[OH]-l[L]+(\text{Sum of } (im+j-kl)[M_iH_jL_k]) = 0$$

At each point in a potentiometric titration experiment, T_M, T_L, [B] and [X] are known. On rearrangement and substitution of the above equations, a set of equations with three unknowns [M], [L] and β's, is obtained.

$$\beta = \text{function } ([M], [L], 10^{-pH})$$

Several data points are used to solve for the unknown constants.

The spectrophotometric method depends upon the fact that when complex formation takes place, the absorption of the new species formed may be considerably different from that of the reactants. In favorable cases, therefore, the measurement of uv and visible spectra allows a direct calculation of the concentration, c, of the new species. The method is based on Beers Law

$$A = \log (I_0/I) = \epsilon cd$$

where I_0/I is the ratio of incident to transmitted light, c is the molar concentration of the absorbing solute, d is the light path lenght in centimeters, and ϵ is the molar absorptivity. The same equilibrium equations as described in the potentiometric method are used for the calculations. The Job method of continuous variation can yield both the

stability constants and stoichiometry of the species.

Ceratenolone

Ceratenolone (1) is isolated from the crude extract of *C.minor* as a violet material. We suggested that this violet color arises *via* complex formation with ferric ions. Equations (1), (2), and (3) illustrate the stepwise formation of mono-, bis-, and tris- complexes respectively. We set out to determine which complex is formed with ceratenolone as the ligand (HL), and to evaluate the various equilibrium constants involved.

(1) $[Fe(H_2O)_6]^{+3} + HL \rightleftharpoons [FeL(H_2O)_4]^{+2} + H^+ + 2\ H_2O$

(2) $[FeL(H_2O)_4]^{+2} + HL \rightleftharpoons [FeL_2(H_2O)_2]^{+} + H^+ + 2\ H_2O$

(3) $[FeL_2(H_2O)_2]^{+} + HL \rightleftharpoons [FeL_3] + H^+ + 2\ H_2O$

Two initial observations give us an idea of the nature of the complex formed and the magnitude of the formation constant. First, the violet colored ceratenolone is very soluble in organic solvents such as hexane and chloroform. If the violet coloration is indeed the result of complexation with iron, then the iron complex must be electrically neutral. Only the tris complex satisfies this requirement. This suggests that ceratenolone forms a tris

complex with ferric ions.

Second, the color of a solution of 1 disappears on treatment with base and does not reappear upon reacidification. Close examination of this process shows that as the pH of the acidic violet solution is raised, the violet color (figure 13; curve A) fades and completely disappears at a pH of 6-7 (curve B). This color loss is accompanied by the formation of a precipitate of colloidal ferric oxide FeO(OH)(am). On reacidification, the Fe ions are not immediately available for rechelating with ceratenolone, thus no violet colored complex is formed (curve C). However, on prolongued exposure to acid, the ferric oxide slowly redissolves and the violet color reappears.

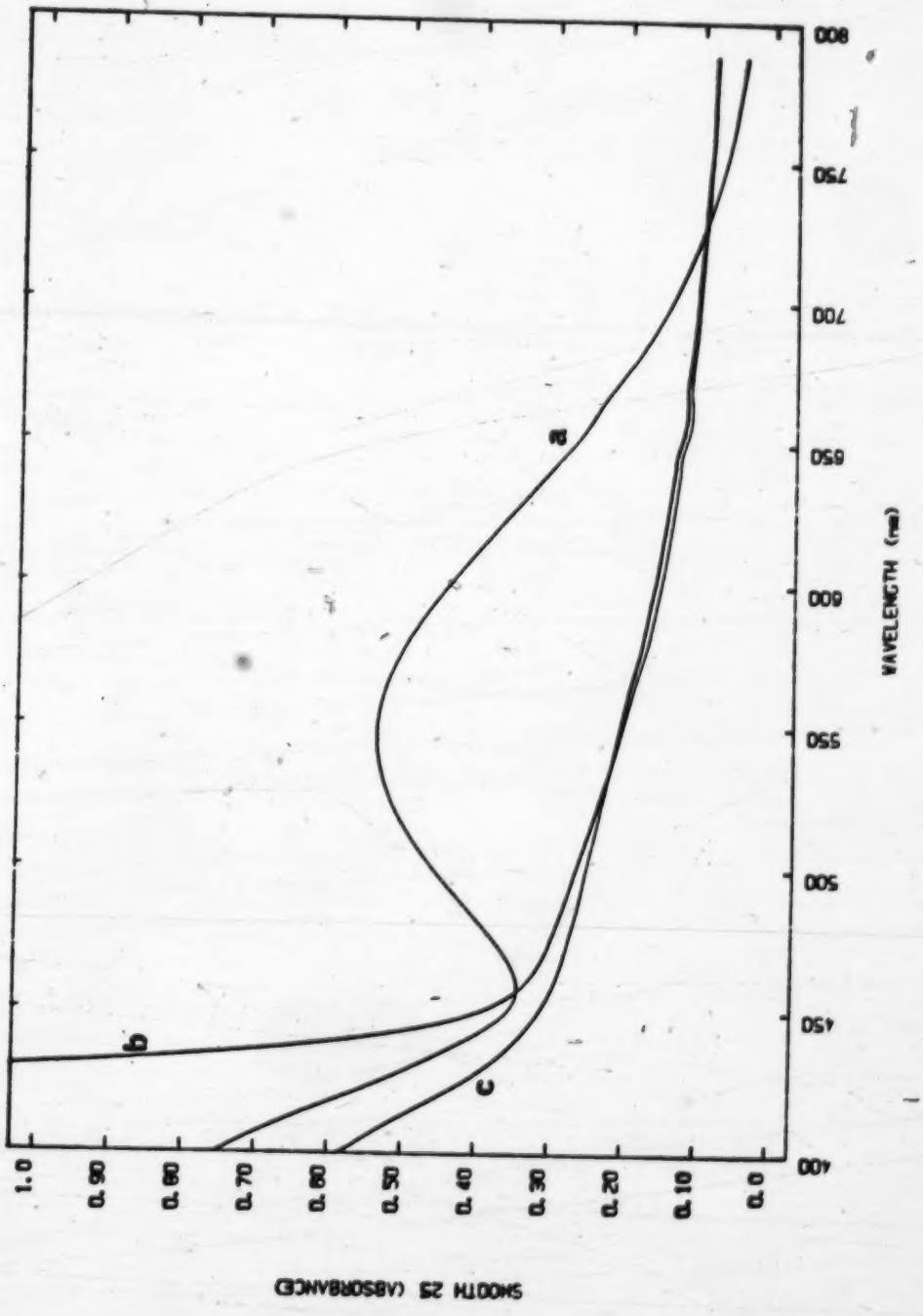
$$Fe^{+3} + 3\ H_2O \rightleftharpoons FeO(OH) + 3\ H^+ + H_2O$$

$$\log K = -3.5$$

This suggests that the overall formation constant for the iron complex is not very large as hydroxide ions can compete with ceratenolone for the ferric ions. Precipitation of ferric oxide rapidly shifts the equilibrium.

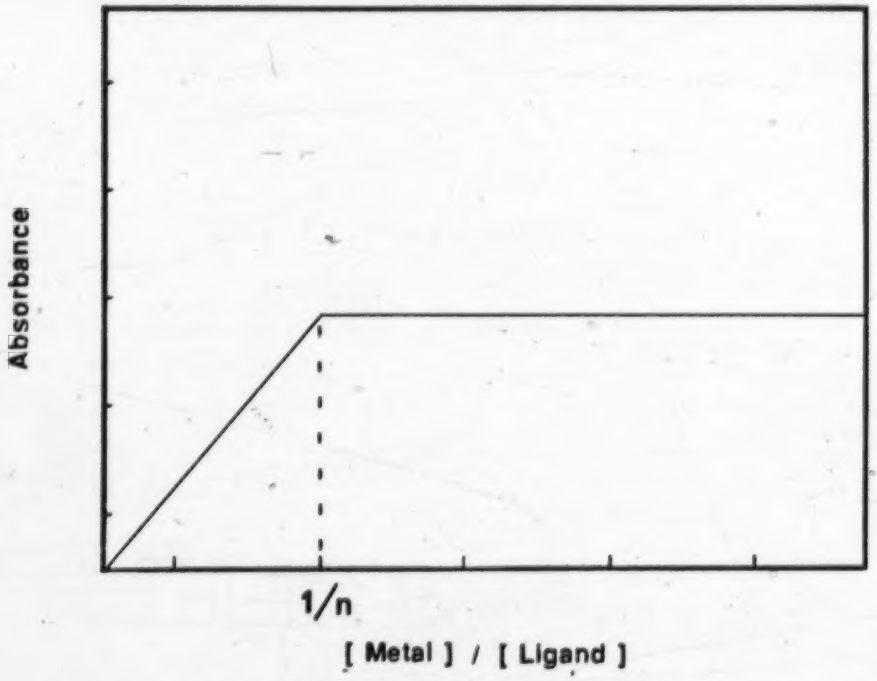
In order to determine the nature of the complex formed with ceratenolone, a solution of ceratenolone was titrated with a solution of ferric ions. A plot of the observed absorbance against the number of equivalents of ferric ions added was expected to give a plot as shown in figure 14,

Figure 13. Visible spectrum of ceratenolone - iron complex



from which the number of ceratenolone molecules (n) involved in the complex can be determined. Thus, for the formation of a tris complex, the absorbance of the solution should rise linearly with added iron, and level off after 0.33 equivalents of ferric ions have been added, corresponding to a 3:1 ratio of ligand to iron.

Figure 14. Expected plot of titration of ceratenolone with iron



As seen in figure 15, the titration curve obtained is quite different from that expected. The absorbance of the solution (pH = 2.61) initially rises linearly and levels off, as expected, after 0.33 equivalents of iron had been added (corresponding to a 3:1 ligand:iron ratio). Subsequent addition of iron brings about a sharp decline in

the absorbance of the solution until 0.66 equivalents of iron have been added. This corresponds to a 1.5:1 ligand:iron ratio. After this point, the absorbance of the solution decreased only slightly with further addition of iron ions.

This experiment can only be interpreted as follows. As iron ions are added to a large excess of ligand, the following reaction must take place :

$$Fe + 3\ L \rightleftharpoons FeL_3$$

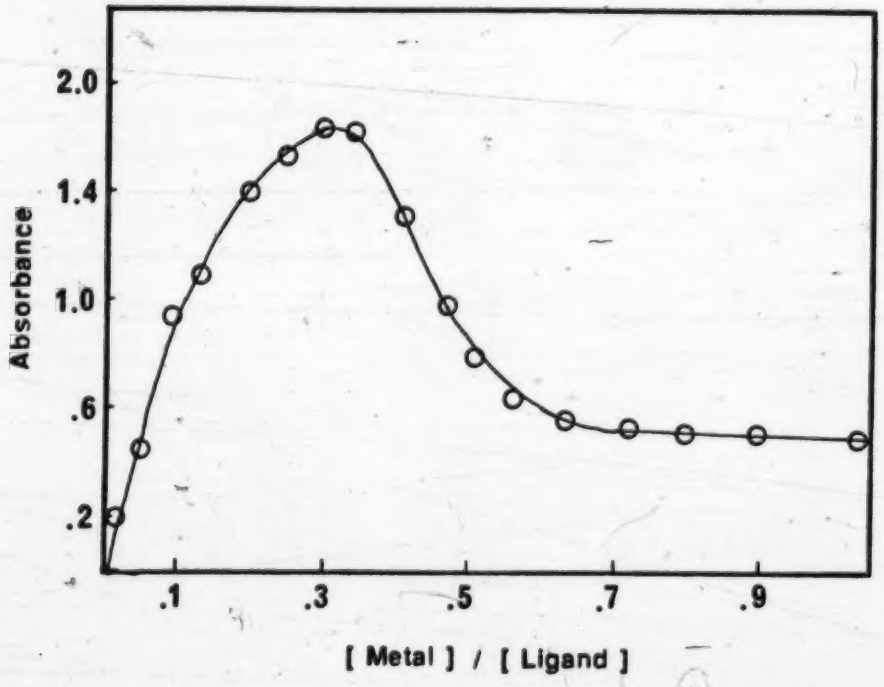
Thus the absorbance increases linearly with addition of iron ions until all the ligands are used in chelation. This point is reached at a 3:1 ratio. The decrease in absorbance upon further addition of iron can be explained by the following reaction, but only if the molar absorbtivity of the FeL_2 and FeL species is significantly less than that of FeL_3 species, and the third formation constant is smaller than the first two.

$$FeL_3 + Fe \rightleftharpoons FeL_2 + FeL$$

Thus, after a second 0.33 equivalent of iron is added, all of the intensely absorbing FeL_3 species has been destroyed, and the absorbance levels off. This explanation, involving a disproportionation, satisfactorily explains the results observed. Throughout the titration, the full visible

spectrum was obtained for each point, and no shift in .lambda.$_{max}$ was observed. It is surprising that the various FeL_n species have the same .lambda.$_{max}$. Indeed, at least with catecholate ligands, there is a very significant shift in the wavelength of maximum absorbancy as the number of chelated species around the iron ion increases (*ca* 140 nm).

Figure 15. Observed plot of titration of ceratenolone with iron



The evidence strongly suggests the formation of a 3:1 ligand:iron complex. This is not so unusual for iron complexes in general, but it is unusual with this type of ligand. For example salicylaldehyde and *o*-hydroxyacetophenone form 1:1 complexes with iron.

Catecholate ligands are usually dissociated to the mono-chelate compounds at high acidities. Thus, it is surprising that ceratenolone forms a tris complex at a pH as low as 2.6 . Furthermore the complex appears to be very stable towards hydrolysis as the violet color, although pale, persists at a pH of 0.9.

In order to confirm the formation of a tris-complex and to obtain the formation constants involved, the reverse titration, i.e. titration of a fixed amount of iron with the ligand, was performed. The result is shown in figure 16.

If one simplifies equations 1-3 to 4-6 to illustrate complex formation, then the formations constants can be written as equations 7-11.

(4) $Fe + L \rightleftharpoons FeL$

(5) $FeL + L \rightleftharpoons FeL_2$

(6) $FeL_2 + L \rightleftharpoons FeL_3$

(7) $K_1 = [FeL]/[Fe][L]$

(8) $K_2 = [FeL_2]/[FeL][L]$

(9) $K_3 = [FeL_3]/[FeL_2][L]$

(10) $\beta_2 = K_1K_2 = [FeL_2]/[Fe][L]^2$

(11) $\beta_3 = K_1K_2K_3 = [FeL_3]/[Fe][L]^3$

(12) $HL \rightleftharpoons H^+ + L^-$

(13) $K_a = [H][L]/[HL]$

From stoichiometric considerations we can write eq. 14 for the total ligand concentration (L_t), and eq. 15 for the

Figure 16. Titration of iron (III) with ceratenolone

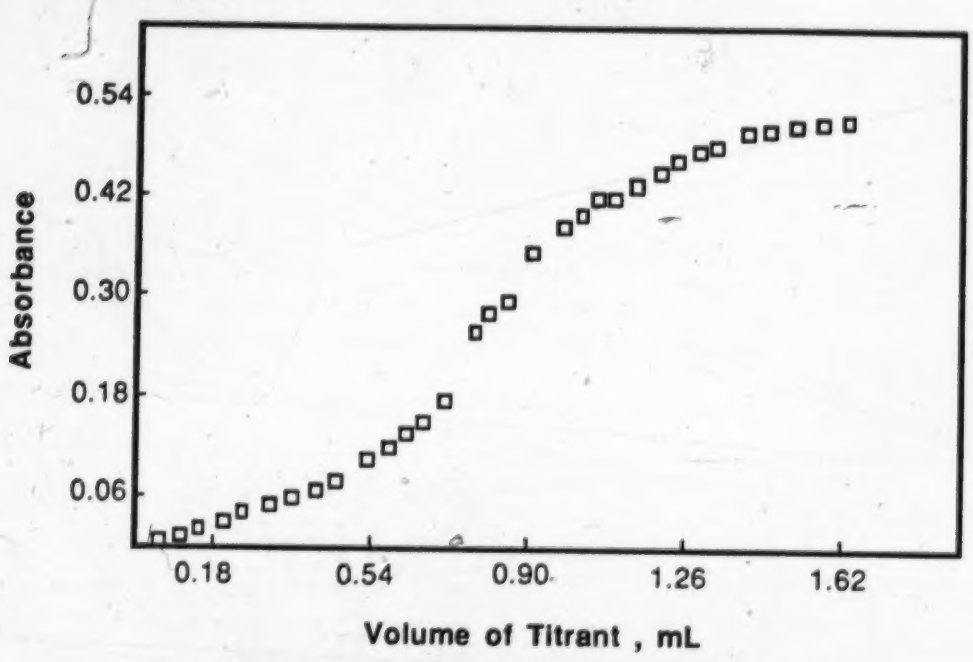
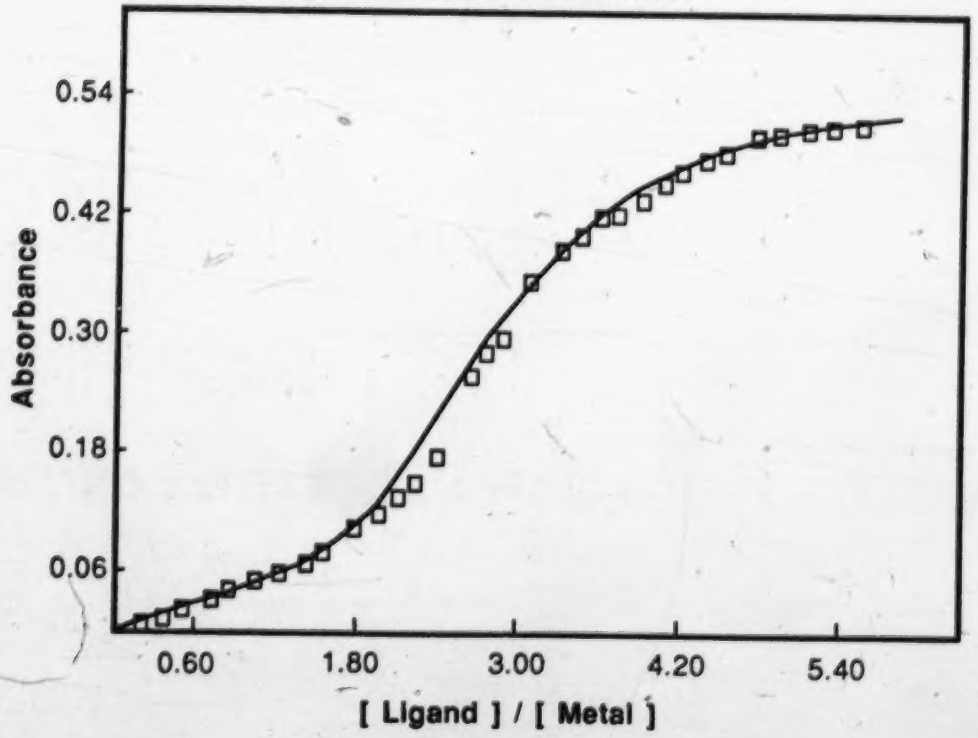


Figure 17. Superimposition of the observed and calculated titration curves of iron (III) with ceratenolone



total iron concentration (Fe_t).

(14) $[L_t] = [HL]+[L]+[FeL]+2[FeL_2]+3[FeL_3]$

(15) $[Fe_t] = [Fe]+[FeL]+[FeL_2]+[FeL_3]$

The absorbance of the solution (A) results from the absorbance of the mono, bis and tris complexes and is related to their concentration by their respective molar absorptivity ϵ_1 , ϵ_2, and ϵ_3, as shown by equation 16.

(16) $A = \epsilon_1[FeL]+\epsilon_2[FeL_2]+\epsilon_3[FeL_3]$

Substitution of 7,10,11,and 13 into 14 and 15 and rearranging, we obtain equation 18 which is a fourth order equation in the concentration of free ligand.

(18) $K_1K_2K_3Q(H/K_a)^4[HL]^4 + K_1K_2(K_3(3Fe_t-L_t)+Q)(H/K_a)^3[HL]^3 + K_1(K_2(2Fe_t-L_t)+Q)(H/K_a)^2[HL]^2 + (K_1(Fe_t-L_t)+Q)(H/K_a)[HL] - L_t = 0$

where $Q = ([H]+K_a)/K_a$

The fourth order equation was solved by iteration for particular K's to calculate [HL] at each point of the titration. This allows the calculation of [Fe], [FeL],[FeL_2] and [FeL_3] for each point. The overall absorbance *versus* titration volume data was then fitted by a non-linear least squares minimization of the error between the calculated and the observed absorbances of the

solution. (The calculations were performed by Dr. Jordan).

Figure 17 shows the calculated curve superimposed with the observed absorbances vs ligand /iron ratios. The good fit further confirms the model used to represent the complexation reaction, i.e., equations 4-6.

The parameters evaluated for the formation constants and the molar absorptivity of the complexes are as follow:

$K_1 = 1 \times 10^8$; $\epsilon_1 = 1000$

$K_2 = 4 \times 10^7$; $\epsilon_2 = 1550$

$K_3 = 1.5 \times 10^6$; $\epsilon_3 = 15700$

$\beta_3 = 6 \times 10^{21}$; $\log\beta_3 = 21.8$

The tris complex has a molar absorptivity ten times greater then the mono and bis complexes and is responsible for most of the coloration. As a result of this dominance of the tris complex, there is some uncertainty for the individual K_1 and K_2 values, but the overall formation constant β_3 remains unaffected.

The distribution curve showing the proportion of the various complex species, at different ligand/iron ratios is shown in figure 18.

This figure shows that at a 3:1 ligand/iron ratio, the predominant species is the bis complex FeL_2^+ and not the tris complex FeL_3 as we had expected. This explains why our attempts at isolating the tris complex by extraction, with hexane or dichloromethane, of a 3:1 ligand/iron

aqueous solution resulted in failure. Indeed, we found that the extract, although violet, consisted mainly of free ligand, as evidenced by its ^1H nmr spectrum. The violet color was produced by a very small proportion (less than 5%) of the tris complex. Furthermore, the isolation of the tris complex now becomes a complicated process. The region where FeL_3 is the predominant species, i.e. ligand/iron ratio greater than 5, also implies the presence of a large excess of free ligand in the organic extract of a solution with such a L/Fe ratio.

The tiration of iron ions with ceratenolone was repeated at a lower pH, (2.01). The result of this titration is shown in figure 19. The fit of the curve using the parameters (K's and ϵ's) calculated previously at pH 2.61, is shown in figure 20. The fit is not as good as before, mainly for points corresponding to the formation of the mono and bis complex species. This deviation can not be easily explained and further studies will be required to explain the results.

It is interesting to compare the formation constant of the ceratenolone - iron complex with those of other ligands. Although it is the conventional form for tabulation, the formation constants are not meaningfull alone in judging the relative ability of ligands to compete with one another at a given pH. This is due to differences in ligand protonation constants (their number and their absolute value), which define the amount of free

Figure 18. Proportion of the various complex species with respect to different ligand / metal ratios

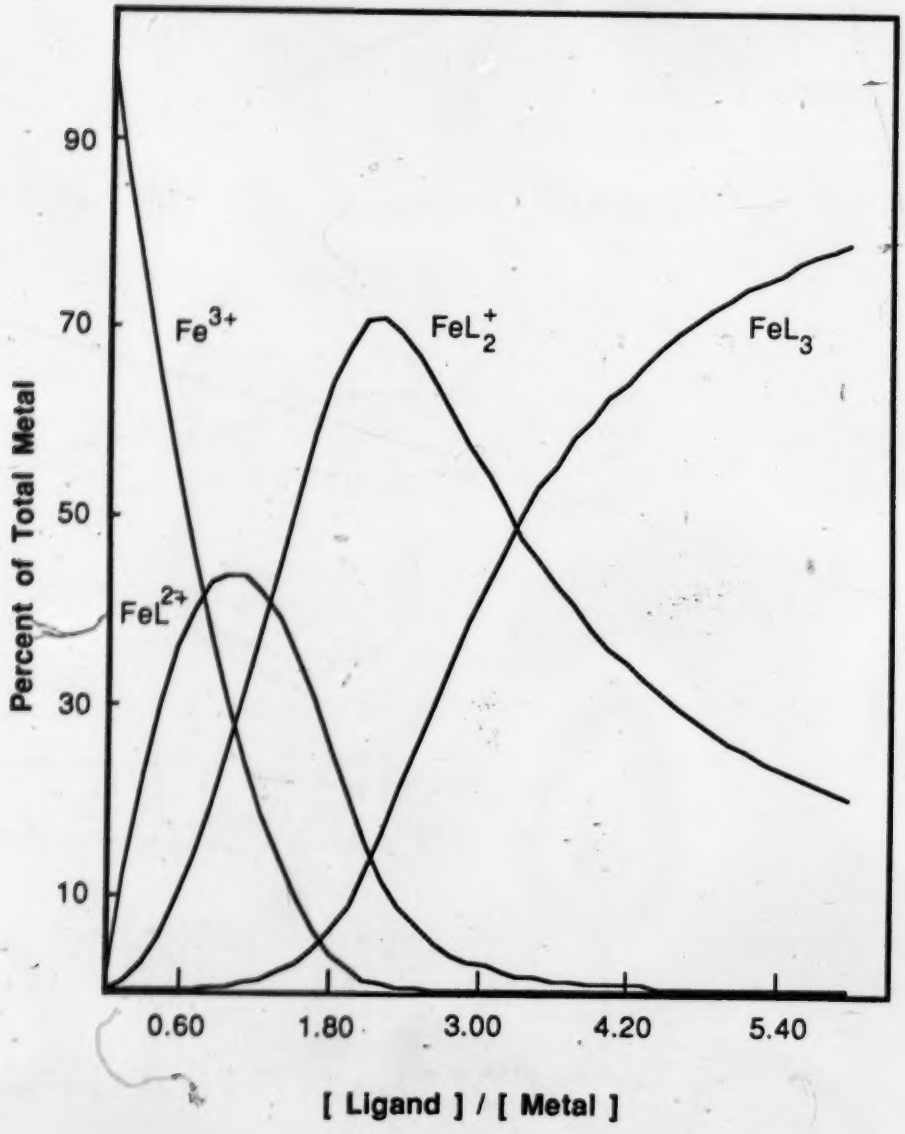


Figure 19. Titration of iron (III) with ceratenolone

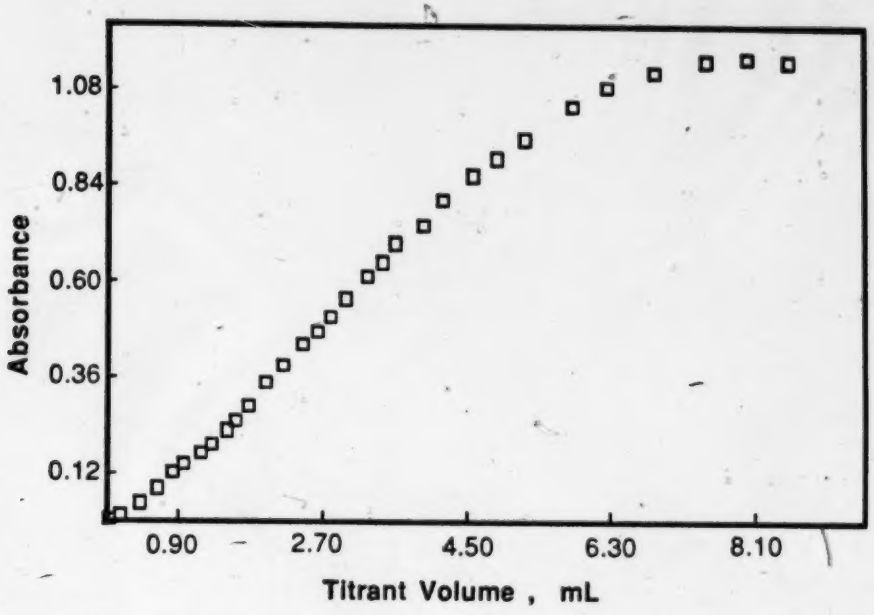
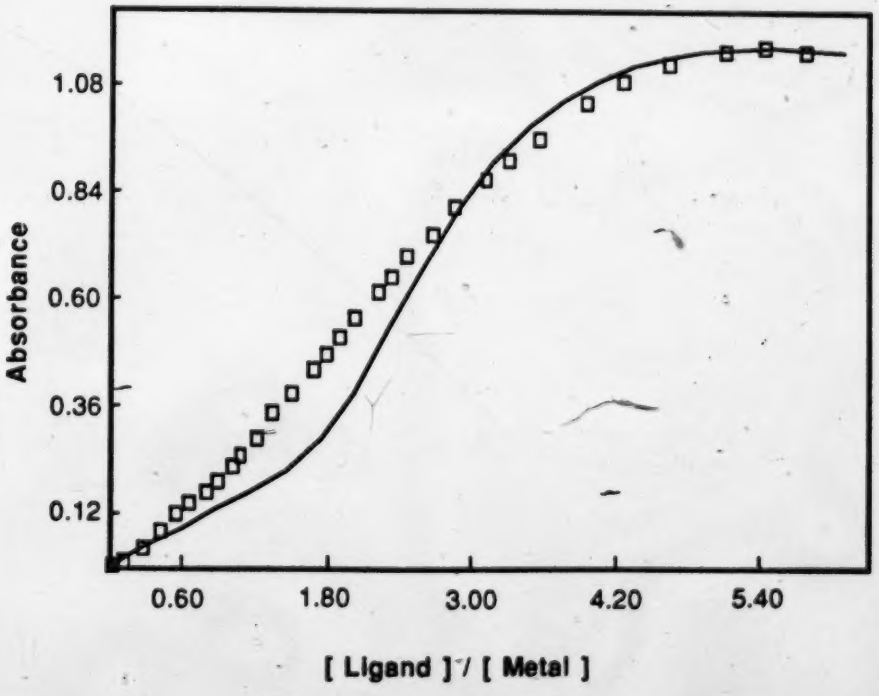


Figure 20. Superimposition of the observed and calculated titration curves of iron (III) with ceratenolone



uncomplexed ligand [L] in aqueous solution. In order to have a more direct ranking of the ligands under specified conditions, so called pM values have been used. pM is defined as $-\log[MH_2O]_n]^{m+}$, the concentration of free metal ion, for physiological conditions, i.e., pH 7.4, ligand concentration 10^{-5}M and metal concentration of 10^{-6}M. The larger the pM the more stable the complex is under the prescribed conditions. Any pM values below the limit set by the K_{sp} of $Fe(OH)_3$ indicates precipitation of ferric hydroxide under these conditions. The calculation of the pM ceratenolone yields a value of 13.3 which is about 5 orders of magnitude below the K_{sp} of $Fe(OH)_3$, and thus precipitation occurs under those conditions, as observed.

As shown in table 6, ceratenolone is a very weak siderophore. Its pM value is two orders of magnitude less than catechol, and 12-16 orders less than the strong siderophores. MECAM is one of the best synthetic siderophores, while entrobactin is the best natural one with a pM value of 35.5.

In summary, our investigations reveal that ceratenolone forms a tris complex with iron. The overall formation constant of this complex is low compared to the formation constants of known siderophores, but it is nevertheless significant. The ceratenolone - iron complex has two important properties: it is electrically neutral and possesses large alkyl groups in its outer sphere. Both of these properties make the complex hydrophobic.

Table 6. Stability constants of ferric complexes.

Siderophore	$\log\beta_3$	pM	ref.
Enterobactin	52	35.5	95
MECAM	46	29.4	96
Ferrichrome	29.1	25.2	97
DNB	43.9	(15)	96
Catechol	43.7	(15.8)	96
Ceratenolone	21.5	(13.3)	

The origin of the blue stain in lodgepole pines infected with *C.minor* may be explained in the following way. Ceratenolone, produced by the fungus in the tree, circulates within the tree until it encounters iron ions. The formation of the hydrophobic tris complex of ceratenolone and iron then results in "oiling out" of the complex from the aqueous medium producing a blue staining effect in the organic tissue of the tree. This hypothesis is supported by the observation that the staining is seen only in very close proximity to the areas infected with the fungus.

A study designed to determine whether iron (or other metal) chelation at the infected site might cause metal ion deficiency in other parts of the tree was performed.[98] We found that the natural variabliity of trace element composition of lodgepole pine is quite large. As a result, within tree differences in element levels, that may be induced by fungal infection, cannot be detected with a high degree of confidence. The role, if any, which the blue stain plays in the etiology of the disease is the topic of continuing studies in our laboratories.

IV. Experimental

A. General

High resolution mass spectra were recorded on an A.E.I. MS-50 mass spectrometer coupled to a DS-50 computer. Chemical ionization mass spectra were recorded on an A.E.I. MS-9 mass spectrometer. Data is reported as m/z (relative intensity). Unless diagnostically significant, peaks with intensities less than 20% of the base peak are omitted. Fourier Transform infrared (ftir) spectra were recorded on a Nicolet FT 7199 interferometer. Ultraviolet (uv) spectra were recorded on a Hewlett Packard 8450A diode array spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (nmr) spectra were measured (in $CDCl_3$ unless otherwise noted) on Bruker WH-200 or WH-400 spectrometers. All nmr measurements employed tetramethylsilane (TMS) as an internal standard and are reported in ppm downfield from TMS (δ values). Each signal is reported for one proton unless otherwise noted. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter and circular dichroism (cd) measurements were made with a Durrum Jasco ORD/UV-5 (SS-20 modification) recording spectrometer. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected.

Analytical and separation techniques

Analytical tlc was carried out on silica gel precoated tlc plates (E.Merck DL-Alufulren, Kieselgel 60 F-254; 0.2 mm thickness). The chromatograms were examined under ultraviolet light (254 or 350 nm). Visualization was completed by dipping the plates in a 5% phosphomolibdic acid solution (20 g of $20MoO_3$ $2H_3PO_4$ $48H_2O$, 2.5 g of cerric sulphate $H_4Ce(SO_4)_4$, in 500 mL of 5% aqueous sulphuric acid), or in a 2% vanillin - sulphuric acid solution (2 g of vanillin in 100 mL of 5% methanolic H_2SO_4), followed by careful charing with a hot plate. Alternatively, the tlc plate was dipped in a 2.5% ferric chloride solution (2.5 g $FeCl_3$ in 100 mL methanol).

Preparative tlc was carried out on silica gel precoated glass plates (20 cm x 20 cm; E.Merck 60 F-254; 0.25 mm thickness). Materials were detected by visualization under an ultraviolet lamp (254 or 350 nm). For flash chromatography, E.Merck silica gel 60 (230 - 400 mesh) was employed.

Droplet countercurrent chromatography was performed using the EYELA Droplet Countercurrent Chromatograph model D.C.C.-300 (Tokyo Rikakikai Co.). The two phase solvent system used (chloroform - methanol - water) was allowed to equilibrate for 24 hours before use.

B. *Mycena citricolor*

Materials

M.citricolor was obtained from Costa Rica and the strain (H2 5348 U.of Alberta microfungus collection) has been deposited with the Biosystematics Research Institute (Agriculture Canada) under accession no. DAOM 191786. It was maintained on coffee plants and the fungus was reisolated as needed to initiate large scale cultures (applies to the isolation of compounds 6 to 39). Water for culturing was distilled in an all glass apparatus prior to use (Corning still Mega-Pure System model MP-6A). Solvents for extraction and purification were distilled prior to use. Difco potato dextrose agar and broth were used to prepare the growth media.

Growth of *M. citricolor*

Unless otherwise noted, to initiate still cultures of the fungus, small fragments of the purified fungus were aseptically transferred to agar plates (potato dextrose) and allowed to grow at room temperature, in a well lighted area, for 10 days. These cultures were blended (Waring blender, aseptic conditions) and 10-20 mL aliquots were used to innoculate Fernbach flasks containing 1000 mL of sterile culture medium (20 g potato dextrose broth, 0.4 g yeast extract / liter water). The flasks were stoppered with foam plugs covered loosely with aluminum foil. The

cultures were allowed to mature in a well lighted and ventilated area without agitation.

Extraction of the metabolites.

After a growth period of 6 to 8 weeks, the mycelial growth was separated from the broth by filtration through cheese cloth. The mycelium was extracted by heating under reflux with ethyl acetate in a Soxhlet apparatus for 24 hours. The broth was stirred with ethyl acetate (1 part ethyl acetate / 3 parts broth) for 30 minutes and separated. This was repeated three times. The combined ethyl acetate extracts were reduced in vacuo to ca. 500 mL, dried over anhydrous $MgSO_4$ and evaporated to yield on average 30 mg of crude extract / liter of broth.

Isolation compounds 3 and 4

M.citricolor was obtained from Costa Rica. The fungus was transferred to an agar plate (potatoe dextrose) and allowed to grow at room temperature for 5 days. This culture was used to innoculate an Erlemeyer flask containing 100 mL of culture medium (5 g dried and sterilized bread crumbs / liter water). After a growth period of 10 days (shake culture), the contents of the flask were aseptically transferred to a fermentor flask containing 10L of culture medium (50 g potatoe dextrose broth / liter water) (New Brunswick Scientific Co.; Magnaferm Fermentor). After a

growth period of 3 weeks, the temperature of the medium increased to *ca* 60° due to a malfunction. The temperature remained at this level for *ca* 1 day, until the accident was noticed. The mycelial growth was separated from the broth by ~~filtration~~ through cheese cloth. The broth was extracted with chloroform (3 x 1 part chloroform / 3 parts broth) for 30 minutes and separated. The combined chloroform extracts were concentrated in *vacuo* to *ca* 500 mL, dried over anhydrous $MgSO_4$ and evaporated to yield 1.6 g of crude extract. The metabolites were purified using centrifugal liquid chromatography (clc) and flash chromatography (chloroform to 5% methanol - chloroform gradient).

(R)-mellein (3)

R_f 0.64 (ethyl acetate - Pet. Ether 50:50);
m.p. 49-51°. (benzene);
ftir (chloroform cast): 2923, 2852, 1672, 1620, 1580, 1469, 1296, 1219 and 1118 cm^{-1};
^1H nmr ($CDCl_3$): 11.0 (br s), 7.40 (dd, 6.0, 7.0), 6.89 (dd, 7.0, 1.0), 6.69 (dd, 6.0, 1.0), 4.75 (sext, 7.0), 2.96 (2H, d, 7.0), 1.50 (3H, d, 7.0);
hreims: m/z calculated for $C_{10}H_{10}O_3$: 178.0630; found 178.0632 (100), 160 (33), 134 (78), 106 (20), 77 (31).

Ethyl (2,4-dihydroxy-3-ethyl-6-methyl)benzoate (4)

R_f 0.23 (chloroform-methanol, 99:1);
ftir (chloroform cast): 3453, 2920, 1634, 1619, 1595, 1320, 1278, 1110 and 780 cm^{-1};
^1H nmr ($CDCl_3$): 6.25 (s), 5.12 (br s, D_2O exch.), 4.43 (2H, q, 7.1), 2.89 (2H, q, 7.4), 2.12 (3H, s), 1.59 (br s, D_2O exch.), 1.43 (3H, t, 7.2), 1.20 (3H, t, 7.5);
hreims: m/z calculated for $C_{12}H_{16}O_4$: 224.1048; found 224.1051 (30), 178 (100), 150 (41), 122 (10).

Isolation of compounds 6, 7, 8, 9, and 10

The crude mycelium extract (1.3 g) was subjected to flash chromatography (chloroform to chloroform-methanol, 70:30, gradient). Fractions eluting with chloroform - methanol, 95:5, gave a sample which was further purified by flash chromatography to afford ergosterol (6), ergosterol peroxyde (7), palmitic acid (9), and linoleic acid (10). Fractions eluting with chloroform - methanol, 80:20, afforded cerevisterol (8).

Ergosterol (6)

R_f 0.31 (chloroform-methanol, 98:2);
mp 154-156°
ftir (chloroform cast): 3400 (br), 2951, 2933, 2869, 1600, 1467, 1378, 1068, 1038, 970, 837, and 801 cm^{-1};

^{1}H nmr ($CDCl_3$): 5.60 (dd, J = 6.0, 2.5 Hz), 5.42 (dd, J = 6.0, 3.0 Hz), 5.23 (m, 2H), 3.67 (m), 2.50 (ddd, J = 14.0, 5.0, 2.0 Hz), 2.31 (m), 2.1 - 1.2 (19H), 1.07 (d, J = 7.0 Hz, 3H), 0.98 (s, 3H), 0.95 (d, J = 7.0 Hz, 3H), 0.89 (d, J = 7.0 Hz, 3H), 0.85 (d, J = 7.0 Hz, 3H), 0.66 (s, 3H);
hreims: m/z calculated for $C_{28}H_{44}O$: 396.3392; found 396.3400 (100), 363 (64), 337 (26), 271 (36), 251 (43), 109 (24), 83 (32), 81 (48).

Ergosterol peroxyde (7)

R_f 0.24 (chloroform-methanol, 93:7);
mp 179-181° (hexane)
ftir (chloroform cast): 3400 (br), 2965, 2872, 1470, 1390, 1080, 1050, 1038, and 980 cm^{-1};
^{1}H nmr ($CDCl_3$): 6.50 (d, J = 8.5 Hz), 6.23 (d, J = 8.5 Hz), 5.18 (m, 2H), 3.97 (m), 2.2 - 0.8 (21H), 1.01 (d, J = 7.0 Hz, 3H), 0.91 (d, J = 7.0 Hz, 3H), 0.90 (s, 3H), 0.84 (d, J = 7.0 Hz, 6H), 0.83 (s, 3H);
hreims: m/z calculated for $C_{28}H_{44}O_3$: 428.3279; found 428.3284 (5), 396 (100), 364 (23), 363 (31), 271 (7), 69 (39).

Cerevisterol (8)

R_f 0.15 (chloroform-methanol, 90:10);

mp: 254-258° (methanol)

ftir (chloroform cast): 3610, 3450, 3310, 2980, 2860, 1659, 1459, 1448, 1383, 1370, 1028, 968, 939, and 865 cm^{-1};

^1H nmr ($CDCl_3$): 5.38 (br d, J = 5.0 Hz), 5.20 (m, 2H), 4.08 (m), 3.64 (bs), 2.2 - 1.0 (14H), 1.02 (s, 3H), 0.99 (d, J = 7.0 Hz, 3H), 0.93 (d, J = 7.0 Hz, 3H), 0.86 (d, J = 7.0 Hz, 3H), 0.84 (d, J = 7.0 Hz, 3H), 0.61 (s, 3H);

hreims: m/z calculated for $C_{28}H_{44}O_2$ (M - H_2O): 412.3341; found 412.3332 (26), 394 (20), 379 (30), 251 (25), 95 (25), 93 (27), 81 (42).

Palmitic acid (9)

R_f 0.48 (chloroform-methanol, 98:2);

ftir (chloroform cast): 2400 - 3600 (br), 2917, 2840, 1701, 1460, 1215, and 1158 cm^{-1};

^1H nmr ($CDCl_3$): 11.2 (br s), 2.35 (t, J = 6.0 Hz, 2H), 2.03 (m, 2H), 1.6 (m, 2H), 1.23 (22H), 0.87 (3H);

hreims: m/z calculated for $C_{16}H_{32}O_2$: 256.2402; found 256.2406 (24), 196 (18), 73 (21), 60 (31), 55 (100).

Linoleic acid (10)

R_f 0.55 (chloroform-methanol, 98:2);

ftir (chloroform cast): 2400 - 3600 (br), 2930, 2860, 1710, 1462, 1280, 940, and 740 cm^{-1};

^{1}H nmr ($CDCl_3$): 11.8 (br s), 5.37 (m, 4H), 2.80 (m 2H), 2.36 (t, J = 6.0 Hz, 2H), 2.04 (m, 4H), 1.6 (m, 2H), 1.32 (22H), 0.94 (vt, 3H);

hreims: m/z calculated for $C_{18}H_{32}O_2$: 280.2394; found 280.2399 (12), 220 (5), 108 (36), 94 (20), 60 (25), 55 (100).

Isolation of citricolic acid (11)

The crude broth metabolites (0.30 g) were dissolved in ethyl acetate (100 mL) and extracted with 5% aqueous sodium carbonate (2 x 50 mL). The aqueous extracts were combined, acidified with 6N HCl and extracted with ethyl acetate (2 x 50 mL). The combined ethyl acetate extracts were dried ($MgSO_4$), and evaporated to yield an oil (0.12 g) which was subjected to flash chromatography (dichloromethane to dichloromethane-methanol, 90:10, gradient) to afford a pale yellow oil (5.2 mg). Recrystallization from hexane/chloroform afforded pure citricolic acid (11).

R_f 0.23 (chloroform-methanol, 90:10);

m.p. 202-205° (chloroform/hexane);

$[\alpha]^{25}_D$ +107° (methanol, c=0.59);

uv (methanol): 217 nm (9900);

cd spectrum: positive Cotton effect at 243 nm;

ftir (chloroform cast): 3440 (br), 2960, 2880, 1740, 1705, 1655, 1610,1590, 1440, 1380, 1180, 1140, 1055, 955 cm^{-1};

^{1}H nmr: see Table 3;

^{13}C nmr: see Table 3;

cims (NH_3): 382 (100, M+18), 365 (13, M+1),

hreims: m/z calculated for $C_{21}H_{30}O_4$: 346.2136; found 346.2136 (0.5), 288 (8), 207 (32), 205 (40), 124 (100), 109 (64), 82 (77), 59 (95).

Reduction of citricolic acid

Sodium borohydride (5 mg) was added to a solution of citricolic acid (11) (2 mg) in methanol (2 mL). After 15 min. stirring, 1 drop of glacial acetic acid was added and the solution was evaporated to dryness. The residue was triturated with 10 mL ether/2 mL water, the organic extract dried ($MgSO_4$) and evaporated. Purification by flash chromatography (5% methanol/chloroform) afforded deoxycitricolic acid (14) as an oil.

R_f 0.61 (chloroform-methanol, 90:10);

ftir (chloroform cast): 3400 (br), 2955, 2924, 2873, 2854, 1737, 1649, 1603, 1456, 1422, 1380, 1271, 1121, 1062, and 987 cm^{-1};

^{1}H nmr (CD_3OD): 5.66 (dd, J = 1.2, 1.0 Hz), 5.62 (dd, J = 15.0, 7.0 Hz), 5.45 (dd, J = 15.0, 8.0 Hz), 4.64 (ddd, J = 10.5, 7.5, 1.0), 3.11 (ddd, J = 11.0, 6.8, 1.2 Hz), 2.49 (dq, J = 7.0, 7.0 Hz), 2.41 (dddd, J = 12.5, 7.5, 4.0, 2.5 Hz), 2.18 (dq, J = 8.0, 7.0 Hz), 2.06 (m), 1.89 (ddd, J = 13.0, 13.0, 4.0 Hz), 1.82 (m), 1.73 (ddd, J = 13.5, 4.0,

2.5), 1.6 (m, 3H), 1.18 (s, 3H), 1.13 (s, 3H), 1.06 (d, J = 7.0 Hz, 3H), 1.02 (d, J = 7.0 Hz, 3H), 0.78 (s, 3H);

cims (NH_3): 366 (100, M+18);

hreims: m/z calculated for $C_{18}H_{24}O_2$: 272.1770; found 272.1778 (7), 191 (26), 124 (100), 109 (48), 82 (58), 59 (65).

Acetylation of citricolic acid

Pyridine (1 mL) was added to a solution of citricolic acid (11) (1 mg) in acetic anhydride (1 mL) . The solution was stirred overnight. The solution was then evaporated under reduced pressure, the residue dissolved in ether, and purified by chromatography (pipette column) to afford acetylcitricolic acid (15) as an oil.

R_f 0.65 (chloroform-methanol, 90:10);

ftir (chloroform cast): 3400 (br), 2971, 2965, 2948, 2919, 1765, 1660, 1450, 1362, 1208, 1148, 1038, and 980 cm^{-1};

^1H nmr: 5.85 (d, J = 2.0 Hz), 5.68 (dd, J = 15.0, 7.0 Hz), 5.51 (ddd, J = 15.0, 8.5, 1.0 Hz), 3.25 (ddd, J = 12.0, 7.0, 2.0 Hz), 2.70 (ddd, J = 14.0, 4.0, 2.5 Hz), 2.56 (dq, J = 7.0, 7.0 Hz), 2.24 (dq, J = 8.5, 7.0 Hz), 2.10 (s, 3H), 1.5-2.1 (m, 7H), 1.25 (s, 3H), 1.20 (s, 3H), 1.11 (d, J = 7.0 Hz, 3H), 1.07 (d, J = 7.0 Hz, 3H), 0.76 (s, 3H);

cims (NH_3): 424 (100, M+18);

lreims: 388 (M^+-H_2O, 0.2), 330 (2.2), 249 (9.5), 205 (46), 189 (19), 124 (100), 109 (42), 82 (36), 59 (58), 43 (57).

Esterification of citricolic acid

Diazomethane (1 mL of an ethereal solution) was added to citricolic acid (11) (*ca.* 0.5 mg) in ether (0.5 mL). After 10 min. the solution was evaporated to yield methyl citricolate (16) as an oil.

R_f 0.56 (chloroform-methanol, 90:10);

uv (methanol): 284 nm;

ftir (chloroform cast): 3400 (br), 2958, 2920, 1722, 1458, 1375, 1040, and 778 cm^{-1};

cims (NH_3): 396 (18, M+18), 379 (100, M+1).

Ozonolysis of deoxycitricolic acid

Ozone was bubbled into a solution of deoxycitricolic acid (14) (2 mg) in dichloromethane (5 mL) at -78° until the solution remained blue. Nitrogen was then bubbled through for 5 min, then the solution was warmed to 0°. methanol (2 mL) was then added followed by sodium borohydride (5 mg) and the solution was stirred for 15 min. The reaction was worked up as for 14 to yield the diol 17.

R_f 0.31 (chloroform-methanol, 93:7);

ftir (methanol cast): 3420 (br), 2920, 1738, 1641, 1060, 990 cm^{-1};

^1H nmr: 5.66 (t, J = 1.5 Hz), 4.65 (ddd, J = 10.0, 8.0, 1.0 Hz), 4.09 (dd, J = 10.8, 2.8 Hz), 3.71 (dd, J = 10.5, 2.5 Hz), 3.15 (ddd, J = 12.0, 7.0, 1.8 Hz), 1.4-2.1 (m, 7H),

1.16 (d, J = 7.0 Hz, 3H), 0.62 (s, 3H);
cims (NH_3): 284 (100, M+18), 267 (7, M+1);
hreims: m/z calculated for $C_{15}H_{22}O_4$: 266.1512; found 266.1516 (19), 248 (13), 230 (11), 207 (13), 189 (18), 164 (45), 151 (100), 119 (50), 105 (46).

Hydrogenation of citricolic acid

Citricolic acid (11) (2 mg) was added to a suspension of 10% Pd/C (5 mg) in 3 mL methanol. A balloon filled with hydrogen gas was attached to the flask and the suspension was stirred for 3 hours. The suspension was filtered through celite and the solvent evaporated under reduced pressure to give dihydrocitricolic acid (18) as an oil.
R_f 0.38 (chloroform-methanol, 90:10);
ftir (chloroform cast): 3400 (br), 2958, 2928, 2873, 2857, 1737, 1649, 1462, 1379, 1275, 1260, 1191, 1163, 1127, 1067, 996, and 939 cm^{-1};
^1H nmr (CD_3OD): 5.73 (br s), 3.18 (m), 2.43 (m), 1.3-2.1 (m, 17H), 1.14 (s, 3H), 1.12 (s, 3H), 1.00 (d, J = 7.0 Hz, 3H), 0.96 (d, J = 7.0 Hz, 3H), 0.69 (s, 3H);
lreims: 332 (20), 317 (16), 307 (11), 238 (14), 218 (20), 181 (45), 152 (31), 151 (42), 97 (38), 96 (73), 59 (100), 55 (69).

Isolation of compounds 30, 31, and 34

The crude broth metabolites were separated by dccc. A chloroform - methanol - water mixture (5:6:4) was prepared and equilibrated. The lower layer of the solvent mixture was used as the mobile phase, and the upper layer as the stationary phase, in a descending mode dccc. The crude extract was dissolved in 20 mL of the upper layer and injected into the injection loop. A flow rate setting of 180 was used which results in a flow rate of 12 mL / hour. 5 mL fractions were collected. Fractions 20 to 50 were combined and the solvent evaporated under reduced pressure. The residue was applied to a flash chromatography column and elution (5% methanol - chloroform) afforded cyclo(L-Pro-L-Leu) (30) and citricolic acid (11). Fractions 51 to 99 were combined and the solvent evaporated under reduced pressure. The residue was applied to a flash chromatography column and elution (2% - 10% methanol - chloroform gradient) afforded hydroxy furans 31 and 34.

cyclo(L-Pro-L-Leu) (30)

R_f 0.62 (chloroform-methanol, 93:7);
ftir (chloroform cast): 3370 (br), 2923, 1731, 1708, 1425, 1311, 1251, 1142, 1198, 1171, 1147, 1075, 1060, and 730 cm^{-1};
^1H nmr ($CDCl_3$): 5.79 (bs), 4.12 (t, J = 8.5 Hz), 4.02 (dd, J = 9.5 and 3.5 Hz), 3.56 (m, 2H), 2.35 (dddd, J = 13.0, 8.0, 7.0, 3.0 Hz), 2.1 (m, 3H), 1.91 (m), 1.73 (m), 1.52

(ddd, J = 13.5, 9.5 and 5.0 Hz), 1.00 (d, J = 6.5 Hz, 3H), 0.95 (d, J = 6.5 Hz, 3H);

cims: 211 (100, M+1), 228 (20, M+18);

hreims: m/z calculated for $C_{11}H_{18}N_2O_2$: 210.1368; found 210.1371 (0.7), 154 (100), 138 (10), 125 (11), 86 (21), 70 (55), 56 (6).

2-hydroxymethyl-5-(4-3-buten-2-one)-furan (31)

R_f 0.29 (chloroform-methanol, 95:5);

ftir (chloroform cast): 3360, 2920, 1682, 1622, 1360, 1263, 1181, 1018 and 966 cm^{-1};

uv (methanol): 320, 212 nm;

^1H nmr ($CDCl_3$): 7.30 (d, J = 15.5 Hz), 6.64 (d, J = 3.5 Hz), 6.63 (d, J = 15.5 Hz), 6.42 (d, J = 3.5 Hz), 4.68 (s, 2H), 2.36 (s, 3H);

hreims: m/z calculated for $C_9H_{10}O_3$: 166.0630; found 166.0633 (22), 151 (9), 135 (100), 107 (8).

5-hydroxymethyl furfural (34)

R_f 0.24 (chloroform-methanol, 95-5);

ftir (chloroform cast): 3420 (br), 1678, 1585, and 1515 cm^{-1};

uv (methanol): 282, 227 nm;

^1H nmr ($CDCl_3$): 9.25 (s), 7.15 (d, J = 3 Hz), 6.47 (d, J = 3 Hz), 4.70 (s, 2H), 4.1 (br s, D_2O exch.);

hreims: m/z calculated for $C_6H_6O_3$: 126.0316; found 126.0319 (73), 97 (100), 69 (33).

Isolation of compounds 35 and 36

The crude broth metabolites (190 mg) were dissolved in ethyl acetate (100 mL) and extracted with 1*N* NaOH (2 x 50 mL). The aqueous extracts were combined, acidified with 6N HCl and extracted with ethyl acetate (2 x 50 mL). The combined ethyl acetate extracts were dried ($MgSO_4$), and evaporated to yield an oil (38 mg). Separation by flash chromatography (dichloromethane to dichloromethane - methanol, 90:10, gradient), afforded 35 as a yellow solid (9.1 mg) and 36 (12.3 mg).

p-hydroxybenzaldehyde (35)

R_f 0.34 (chloroform-methanol, 93:7);
mp 116-118° (chloroform)
ftir (chloroform cast): 3580, 3019, 2925, 2854, 2355, 1687, 1602, 1587 and 1159 cm^{-1};
^1H nmr ($CDCl_3$): 9.87 (s), 7.80 (d, J = 8.5 Hz, 2H), 6.96 (d, J = 8.5 Hz, 2H);
hreims: m/z calculated for $C_7H_6O_2$: 122.0368; found 122.0370 (87), 121 (100), 93 (35).

tyrosol (36)

R_f 0.10 (chloroform-methanol, 93:7);

ftir (chloroform cast): 3394, 3155, 1515, 1052, 818 and 556 cm^{-1};

^1H nmr ($CDCl_3$): 7.01 (dt, J = 8.0 and 1.0 Hz, 2H), 6.71 (dt, J = 8.0 and 2.0 Hz, 2H), 3.72 (t, J = 6.5 Hz, 2H), 2.72 (t, J = 6.5 Hz, 2H), 2.52 (bs, 2H);

hreims: m/z calculated for $C_8H_{10}O_2$: 138.0681; found 138.0679 (25), 107 (100), 77 (11).

Isolation of compounds 38 and 39

5 L of broth was extracted with dichloromethane (2 x 500 mL dichloromethane / 1 L broth). The broth was filtered through celite and passed through an Amberlite resin (XAD-4) column at a flow rate of 150 mL / hour (pump control). The column was then eluted with 25% methanol - water (250 mL), followed by 100% methanol (400 mL). Evaporation of the methanol eluant, gave a brown residue (0.775 g). Flash chromatography (10% methanol - chloroform) on a portion of the residue (0.336 g) afforded 39. The remainder of the brown residue was dissolved in pyridine (5 mL) and acetic anhydride (5 mL). The solution was stirred at room temperature for 24 hours. The solution was evaporayed to dryness (Buchi evaporator) and the residue disolved in dichloromethane. Purification by flash chromatography (4% methanol - chloroform) afforded acetylated-39 and 38.

2,3-dihydroxybutane (39)

R_f 0.18 (chloroform-methanol, 93:7);
ftir (chloroform cast): 3340, 2975, 2933, 2891, 1453, 1373, 1079, 1056, 1005 and 888 cm^{-1};
^1H nmr ($CDCl_3$): 3.78 (bs, 1H, D_2O exch.), 3.65 (q, J = 6.5 Hz, 2H), 1.00 (d, J = 6.5 Hz, 6H);
^{13}C nmr ($CDCl_3$): 70.56, 16.61;
hreims: m/z calculated for C_4H_8O ($M-H_2O$): 72.0575; found 72.0578 (16), 45 (100).

2,3-diacetoxybutane

R_f 0.86 (chloroform-methanol, 93:7);
ftir (chloroform cast): 2960, 2920, 1741, 1371, 1232 and 1043 cm^{-1};
^1H nmr ($CDCl_3$): 5.00 (q, J = 6.6 Hz, 2H), 2.03 (s, 6H), 1.18 (d, J = 6.6 Hz, 6H);
cims: m/z 192 (100, M+18);
lreims: 131 (4), 114 (4), 87 (22), 43 (100).

3-hydroxy-γ-butyrolactone acetate (38)

R_f 0.76 (chloroform-methanol, 93:7);
ftir (chloroform cast): 1785, 1740, 1180, 1095, 1048, 1030, and 990 cm^{-1};

^1H nmr ($CDCl_3$): 5.45 (dddd, J = 6.5, 4.5, 2.0, 1.5 Hz), 4.55 (dd, J = 11.0, 4.5 Hz), 4.40 (ddd, J = 11.0, 1.5, 1.5 Hz), 2.90 (dd, J = 18.0, 6.5 Hz), 2.65 (ddd, J = 18.0, 2.0, 1.5 Hz), 2.13 (s, 3H);

hreims: m/z calculated for $C_6H_8O_4$: 144.0423; found 144.0425 (0.8), 86 (20), 84 (100), 71 (11), 55 (14).

C. Synthesis of ceratenolone

Reagents and solvents

Anhydrous solvents and reagents were distilled from appropriate drying agents (in brackets) under an inert atmosphere of nitrogen or argon: THF (potassium), acetonitrile (calcium hydride), *tert*-butyl alcohol (potassium), ethyl formate (phosphorous pentoxide), benzene (sodium), pyridine (calcium hydride), diisopropylamine (calcium hydride), triethylamine (calcium hydride), chlorotrimethylsilane (calcium hydride), ethanol (sodium). Other reagents were used without further purification.

5-Methyl-3-hepten-2-one (59)

Sodium methoxide (1.62 g, 30 mmol) was added to dry methanol (50 mL) followed by phosphonate 61 (5.82 g, 30 mmol). After one min 2-methylbutyraldehyde (48) (3.2 mL, 30 mmol) was added dropwise. The solution was stirred for 30 min, then poured into a mixture of water (50 mL) and pentane (50 mL). The aqueous layer was separated then extracted with pentane (2 x 50 mL). The organic extracts were then combined, washed with brine, dried ($MgSO_4$), and evaporated to give enone 59 (3.53 g, 93%) as a colorless oil;

R_f 0.47 (dichloromethane);

ftir (chloroform cast): 2964, 2933, 2876, 1670, 1639, 1460, 1380, 1175, and 981 cm^{-1};

^1H nmr ($CDCl_3$): 6.66 (dd, J = 16.0, 8.0 Hz, 1H, C-4 C*H*), 6.02 (dd, J = 16.0, 1.5 Hz, 1H, C-3 C*H*), 2.24 (br sept, J = 7.0 Hz, 1H, C-5 C*H*), 2.23 (s, 3H, C-1 CH_3), 1.42 (quint, J = 7.0 Hz, 2H, C-6 CH_2), 1.05 (d, J = 7.0 Hz, 3H, C-8 CH_3), 0.87 (t, J = 7.0 Hz, 3H, C-7 CH_3);

hreims: m/z calculated for $C_8H_{14}O$: 126.1045; found 126.1043 (38), 111 (100), 83 (22).

5-(1-methylpropyl)-1,3-cyclohexanedione (58)

In a dry 100 mL three-necked round bottomed flask, fitted with a reflux condenser, under an argon atmosphere, was placed absolute ethanol (40 mL). Clean sodium (0.71g, 31 mmol) was then added at such a rate that the reaction was

kept at the boiling temperature. After the sodium had dissolved completely, diethyl malonate (4.7 mL, 31 mmol) was added and then a solution of enone 59 (3.53g, 28 mmol) in absolute ethanol (5 mL) was added slowly *via* syringue. The solution was refluxed with constant stirring for two hours, after which a solution of potassium hydroxide (17.5 mL of a 3.9*N* solution) was added and the mixture refluxed for a further six hours. The mixture was cooled, water (50 mL) was added, and the solution was neutralized to pH 7.0 with 1*N* HCl. Most of the ethanol was removed by distillation under reduced pressure (Buchi evaporator). The aqueous solution was acidified with 1*N* HCl and gently heated to complete decarboxylation. After cooling, the mixture was extracted with dichloromethane (3 x 100 mL), the organic extracts combined, washed with brine (50 mL), dried ($MgSO_4$), and evaporated to yield crude dione 58 as a yellow solid. Purification by flash chromatography afforded pure dione (3.26g, 19.4 mmol; 69%).

R_f 0.17 (dichloromethane-methanol, 95:5);

ftir (chloroform cast): 2963, 2938, 2877, 1602 (s), 1414, 1266, and 1101 cm^{-1};

^1H nmr ($CDCl_3$): 8.7 (br s, enol O*H*), 5.48 (s, enol, C-2 C*H*), 3.40 (s, keto, C-2 CH_2), 2.3 - 2.5 (m, 4H, C-4 and C-6 CH_2's), 2.0 (m, 1H, C-5 C*H*), 1.4 (m, 2H, C-8 CH_2), 1.2 (m, 1H, C-7 C*H*), 0.9 (m, 6H, C-9 and C-10 CH_3's);

^{13}C nmr ($CDCl_3$): 203.99 and 203.87 (C-1 and C-3), 104.41 (C-2 enol), 57.58 (C-2 keto), 44.75 and 43.09 (C-4 and

C-6), 38.32 and 38.11 (C-5), 35.38 and 35.14 (C-7), 26.37 (C-8), 15.15 (C-10), 11.39 (C-9);

hreims: m/z calcd. for $C_{10}H_{16}O_2$: 168.1150; found 168.1149 (19), 111 (100), 110 (21), 83 (89), 69 (37), 55 (69).

2-ethyl-5-(1-methylpropyl)-1,3-cyclohexanedione (52): from 58

Sodium hydroxide (185 mg, 4.6 mmol) in water (8 mL) was added to a solution of dione 58 (650 mg; 3.87 mmol) in dioxane (2 mL), under an argon atmosphere. Iodoethane (1.5 mL, 18.7 mmol) was then added and the mixture heated to reflux. After 24 hours, the mixture was cooled, water (25 mL) was added, and the solution was extracted with dichloromethane (3 x 25 mL). The combined organic extracts were washed with brine (2 x 10 mL), dried ($MgSO_4$), and evaporated to afford a brown oil (530 mg). Purification by flash chromatography afforded 3-ethoxy- 5-(1-methylpropyl)-2-cyclohexenone (73) as the major component (309 mg, 1.58 mmol; 41%). Acidification of the aqueous layer with 1*N* HCl afforded a white precipitate. The precipitate was filtered and dissolved in dichloromethane. The dichloromethane solution was dried ($MgSO_4$) and evaporated to yield a white solid (210 mg). Purification by flash chromatography afforded pure dione 52 (158 mg, 0.81 mmol; 19%):

R_f 0.46 (dichloromethane-methanol, 95:5);

mp: 102-104° (Et_2O);

ftir (chloroform cast): 3200-2100 (br), 2962, 2934, 2877, 1565 (s), 1416, 1260, 1242 and 1103 cm^{-1};

^1H nmr ($CDCl_3$): 7.2 (br s, enol OH), 3.28 and 3.25 (t, J = 7.0 Hz, keto, C-2 CH *cis* and *trans*), 2.30 (q, J = 7.0 Hz, enol, C-11 CH_2), 2.3 - 2.5 (m, 4H, C-4 and C-6 CH_2's), 2.0 (m, 1H, C-5 CH), 1.83 (m, keto, C-11 CH_2), 1.4 (m, 2H, C-8 CH_2), 1.2 (m, 1H, C-7 CH), 0.97 (t, J = 7.0 Hz, enol, C-12 CH_3), 0.9 (m, C-9, C-10 and keto C-12 CH_3's);

^{13}C nmr ($CDCl_3$): 206.34 and 204.65 (C-1 and C-3 keto *cis* and *trans*), 185.67 (C-1 and C-3 enol *cis* and *trans*), 117.28 (C-2 enol), 68.29 and 67.61 (C-2 keto *cis* and *trans*), 45.37 and 42.10 (C-4 and C-6 keto *cis* and *trans*), 43.60 (C-4 and C-6 enol), 38.35 (C-5), 37.68 (C-7), 29.29 (C-11 enol), 26.29 (C-8), 15.48 (C-10), 18.87 and 15.01 (C-11, keto *cis* and *trans*), 15.26 (C-12, keto), 11.44 (C-9), 13.08 (C-12, enol);

hreims: m/z calcd. for $C_{12}H_{20}O_2$: 196.1463; found 196.1465 (31), 111 (100), 69 (29), 55 (55).

3-ethoxy- 5-(1-methylpropyl)- 2-cyclohexenone (73):

R_f 0.59 (dichloromethane-methanol, 95:5);

mp: 39-40°;

ftir (chloroform cast): 2962, 2934, 2876, 1657, 1607, 1378, 1353, 1211 and 1143 cm^{-1};

^{1}H nmr ($CDCl_3$): 5.38 (s, 1H, C-2 *CH*), 4.92 (q, J = 7.0 Hz, 2H OCH_2CH_3), 2.3 - 2.5 (m, 4H, C-4 and C-6 CH_2's), 2.0 (m, 1H, C-5 *CH*), 1.4 (m, 2H, C-8 CH_2), 1.39 (t, J = 7.0 Hz, 3H, OCH_2CH_3), 1.2 (m, 1H, C-7 *CH*), 0.9 (m, 6H, C-9 and C-10 CH_3's);

hreims: m/z calcd. for $C_{12}H_{20}O_2$: 196.1463; found 196.1467 (26), 139 (100), 125 (25), 112 (36), 111 (38), 110 (41), 98 (25), 84 (83), 68 (80), 55 (36).

7-Methyl-5-nonen-4-one (54)

N-butyllithium (28.8 mL of a 1.55*N* solution in hexanes, 45 mmol) was added to a solution of diisopropylamine (9.4 mL, 67 mmol) in dry THF (100 mL) under argon at -78°. The solution was stirred for 30 min then 2-pentanone (56) (4.7 mL, 45 mmol) was added dropwise. This solution was stirred for 60 min and 2-methylbutyraldehyde (48) (5.0 mL, 47 mmol) was rapidly added. After stirring for a further 10 min at -78 , sat. aq. NH_4Cl (5 mL) was added. The solution was allowed to warm to room temperature and was poured into a mixture of cold 10% HCl (200 mL) and diethyl ether (100 mL). The organic layer was separated, washed with water, brine, dried ($MgSO_4$), and the solvent evaporated under reduced pressure to give the crude *β*-hydroxyketone 95 (R_f: 0.20 dichloromethane).

The crude *β*-hydroxyketone was dissolved in benzene (100 mL) and *p*-toluenesulphonic acid (1 g) was added. After

stirring for 15 min, 4A molecular sieves (30 g) were added and the mixture was allowed to stand at room temperature for 24 hours. The resulting brown solution was filtered and washed with 5% $NaHCO_3$, water, brine, dried ($MgSO_4$), and the solvent evaporated to give the crude enone (6.5 g). Purification by flash chromatography afforded pure enone 54 (5.9 g, 78%);

R_f 0.61 (dichloromethane);

ftir (chloroform cast): 2963, 2934, 2875, 1675, 1631, 1568, 1459, 1378, 1194 and 981 cm^{-1};

^1H nmr ($CDCl_3$): 6.81 (dd, J = 16.0, 8.0 Hz, 1H, C-6 CH), 6.04 (dd, J = 16.0, 1.5 Hz, 1H, C-5 CH), 2.51 (t, J = 7.0 Hz, 2H, C-3 CH_2), 2.20 (br sept, J = 7.0 Hz, 1H, C-7 CH), 1.61 (sext, J = 7.0 Hz, 2H, C-2 CH_2), 1.30 (quint, J = 7.0 Hz, 2H, C-8 CH_2), 1.04 (d, J = 7.0 Hz, 3H, C-10 CH_3), 0.92 (t, J = 7.0 Hz, 3H, C-11 CH_3), 0.87 (t, J = 7.0 Hz, 3H, C-9 CH_3);

^{13}C nmr ($CDCl_3$): 199.34 (C-4), 151.04 (C-6), 128.45 (C-5), 41.58 (C-3), 37.75 (C-7), 28.51 (C-8), 18.44 (C-10), 17.27 (C-2), 10.91 (C-1 and C-9);

hreims: m/z calculated for $C_{10}H_{18}O$: 154.1357; found 154.1355 (17), 126 (67), 111 (61), 83 (39), 71 (100), 55 (60).

3-(1-methylpropyl)-5-oxooctanoic acid (76)

In a dry 25 mL three-necked round bottomed flask, fitted

with a reflux condenser, under an argon atmosphere, was placed absolute ethanol (8 mL). Clean sodium (41 mg, 1.8 mmol) was added. After the sodium had dissolved completely, diethyl malonate (0.28 mL, 1.8 mmol) was added then a solution of enone 54 (245 mg, 1.6 mmol) in absolute ethanol (1 mL) was added slowly *via* syringue. The solution was refluxed with constant stirring for two hours, after which a solution of potassium hydroxide (2 mL of a 3.9*N* solution) was added and the mixture refluxed for a further six hours. The mixture was cooled and water (10 mL) was added. The solution was neutralized to pH 7.0 with 1*N* HCl. Most of the ethanol was removed by distillation under reduced pressure (Buchi evaporator). The aqueous solution was acidified with 1*N* HCl and gently heated to complete decarboxylation. After cooling, the mixture was extracted with dichloromethane (3 x 30 mL), the organic extracts combined, washed with brine (25 mL), dried ($MgSO_4$), and evaporated to yield a brown oil. Purification by flash chromatography afforded pure acid 76 (246 mg, 1.1 mmol; 72%).

R_f 0.16 (ethyl acetate);

ftir (chloroform cast): 2300-3500 (br), 2963, 2934, 2876, 1734, 1704, 1464, 141, 1381, and 1126 cm^{-1};

^1H nmr ($CDCl_3$): 10.0 (br s, COO*H*), 2.3-2.5 (m, 6H, C-2, C-4 and C-6 CH_2's), 1.59 (sext, J= 7.0 Hz, 2H, C-7 CH_2), 1.4 (m, 2H, C-10 CH_2), 1.11 (m, 1H, C-9 C*H*), 0.78-0.95 (m, 9H, C-8, C-11, and C-12 CH_3's);

hreims: m/z calcd. for $C_{12}H_{22}O_3$: 214.1569; found 214.1565 (4), 171 (37), 157 (18), 128 (41), 111 (31), 87 (27), 86 (33), 83 (42), 71 (100), 58 (48).

2-Ethyl-4-carboethoxy-5-(1-methylpropyl)-1,3-cyclohexanedione (78)

In a dry 250 mL three-necked round bottomed flask, fitted with a reflux condenser, under an argon atmosphere, was placed absolute ethanol (80 mL). Clean sodium (0.71g, 31 mmol) was added at such a rate that the reaction was kept at the boiling temperature. After the sodium had dissolved completely, diethyl malonate (4.7 mL, 31 mmol) was added and a solution of enone 54 (4.31g, 28 mmol) in absolute ethanol (10 mL) was added slowly *via* syringue. The solution was refluxed with constant stirring for 24 hours. The mixture was cooled and water (50 mL) was added. The solution was neutralized to pH 7.0 with 1*N* HCl. Most of the ethanol was then removed by distillation under reduced pressure (Buchi evaporator). The aqueous solution was acidified with 1*N* HCl and extracted with dichloromethane (3 x 100 ml). The organic extracts were combined, washed with brine (50 mL), dried ($MgSO_4$), and evaporated to yield crude dione ester as a yellow solid. Purification by flash chromatography then afforded pure dione ester 78 as a white solid (6.30g, 23.5 mmol; 84%).

R_f 0.39 (dichloromethane-methanol, 95:5);

mp: 52-54°;

ftir (chloroform cast): 3100(b), 2963, 1733, 1598, 1561, 1394, 1313, 1262, 1110 and 1094 cm^{-1};

^1H nmr ($CDCl_3$): 8.9 (br s, 1H, enol, OH), 4.25 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 3.36 (d, J = 11.0 Hz, 1H, C-4 CH), 2.45 (m, 2H, C-6 CH_2), 2.31 (q, J = 7.0 Hz, 2H, C-7 CH_2), 1.4 (m, 1H, C-5 CH), 1.3 (m, 2H, C-11 CH_2), 1.30 (t, J = 7.0 Hz, 3H, OCH_2CH_3), 1.00 (m, 1H, C-10 CH), 0.94 (t, J = 7.0 Hz, 3H, C-8 CH_3), 0.90 (m, 6H, C-12 and C-13 CH_3's);

^{13}C nmr ($CDCl_3$): 171.36 (C-9), 171.20 (C-1 and C-3), 116.91 (enol C-2), 61.00 (OCH_2CH_3), 40.89 (C-4), 39.06 (C-5), 35.68 (C-10), 27.16 (C-7), 24.23 (C-11), 13.96 (OCH_2CH_3), 13.69 (C-13), 12.82 (C-8), 11.77 (C-12);

hreims: m/z calcd. for $C_{15}H_{24}O_4$: 268.1674; found 268.1676 (6), 211 (29), 139 (28), 115 (44), 111 (44), 105 (55), 87 (100).

2-ethyl-5-(1-methylpropyl)-1,3-cyclohexanedione (52): from 78

Dione ester 78 (2.11 g, 7.8 mmol) was dissolved in 95% ethanol (30 mL) and potassium hydroxide (10 mL of a 3.9*N* solution) was added. The solution was allowed to reflux and stir overnight (*ca* 18 hours) under an argon atmosphere. The reaction mixture was cooled and the ethanol was removed by distillation (Buchi evaporator). Dilute HCl (20 mL of a

5% solution) was added and the solution allowed to reflux for one hour under an argon atmosphere. On cooling, a precipitate formed. The mixture was extracted with dichloromethane (3 x 50 mL). The combined organic extracts were extracted with dil. NaOH (3 x 50 mL of 1*N* solution). The combined aqueous layers were poured into ice cold HCl (200 mL of a 1*N* solution) resulting in the formation of a white precipitate. The precipitate was filtered, dissolved in dichloromethane, and dried ($MgSO_4$). After evaporation of the solvent, the crude dione 52 was obtained and purified by flash chromatography (1.49g, 7.6 mmol; 97%).

2-ethyl-5-(1-methylpropyl)-1,3-cyclohexanedione (52): from 54 and 82

Enone 54 (2.71 g, 17.6 mmol) was added to a suspension of potassium monoethyl malonate 82 (2.8 g, 17.7 mmol) in absolute ethanol (50 mL). Potassium *tert*-butoxide (1.98g, 17.7 mmol) was added. The solution was allowed to reflux for 24 hours. The mixture was cooled and water (50 mL) was added. The solution was neutralized to pH 7.0 with 1*N* HCl. Most of the ethanol was removed by distillation under reduced pressure (Buchi evaporator). The aqueous solution was acidified with 1*N* HCl and gently heated to complete decarboxylation. After cooling, the mixture was extracted with dichloromethane (3 x 100 mL), the organic extracts combined, washed with brine (50 mL), dried ($MgSO_4$), and

evaporated to yield a brown oil. Separation by flash chromatography afforded pure dione 52 (532 mg, 1.79 mmol; 10%), unreacted enone 54 (712 mg, 4.6 mmol; 26%), and several unidentified products.

3-(1-methylpropyl)-2-carboethoxy-5-oxooctanoic acid (91)

Enone 54 (6.04 g, 39.2 mmol) was added to a solution of magnesium monoethyl malonate (6.65 g, 43 mmol) in fresly distilled absolute ethanol (50 mL) . The solution was warmed to 60° and stirred for 15 hours. The cooled solution was poured into a mixture of cold 5% aqueous KOH (100 mL) and ether (100 mL). The aqueous layer was separated, acidified with cold 10% HCl, then extracted with ether (3 x 80 mL). The combined ether extracts were washed with water, brine, dried ($MgSO_4$), and the solvent evaporated under reduced pressure to give the crude acid 91 (3.10 g, 12.9 mmol, 33%; 81% based on recovered enone). Evaporation of the first ether extract afforded unreacted enone 54 (3.60 g, 23.3 mmol; 41%). Purification by flash chromatography provided an analytical sample of acid 91.
R_f 0.10 (dichloromethane-methanol, 98:2);
ftir (chloroform cast): 2400-3400 (br), 2964, 2936, 2877, 1750 (sh), 1734, 1712, 1412, 1380, 1300, 1278, 1258, 1237, 1176 and 1161 cm^{-1};

^{1}H nmr ($CDCl_3$): 11.30 (br s, 1H, COO*H*), 4.21 (m, 2H, OCH_2CH_3), 3.66 (m, 1H, C-2 C*H*), 2.6 (m, 3H, C-3 C*H* and C-4 CH_2), 2.42 (t, J = 7.0 Hz, 2H, C-6 CH_2), 1.58 (sext, J = 7.0 Hz, 2H, C-7 CH_2), 1.4 (m, 2H, C-11 CH_2), 1.28 (t, 3H, OCH_2CH_3), 1.1 (m, 1H, C-10 C*H*), 0.90 (m, 9H, C-8, C-12 and C-13 CH_3's);

cims: 304 (M+18, 100%);

hreims: m/z calculated for $C_{14}H_{26}O_3$ ($M-CO_2$): 242.1882; found 242.1879 (2), 111 (32), 71 (100).

Ethyl 3-(1-methylpropyl)-5-oxooctanoate (92)

The crude acid 91 (3.1 g) was placed in a 5 mL flask and heated to 160° in an oil bath for 15 min, under an argon atmosphere. After cooling the oil to 25°, the crude ester was purified by flash chromatography to give keto ester 92 as a colorless oil (2.71 g, 11.2 mmol; 87%).

R_f 0.69 (dichloromethane-methanol, 98:2);

ftir (chloroform cast): 2962, 1734, 1715, 1461, 1374, 1175 and 1038 cm^{-1};

^{1}H nmr ($CDCl_3$): 4.01 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 1.95-2.4 (m, 5H, C-3 C*H*, C-2 and C-4 CH_2's), 2.30 (t, J = 7.0 Hz, C-6 CH_2), 1.50 (sext, J = 7.0 Hz, 2H, C-7 CH_2), 1.30 (m, 2H, C-10 CH_2), 1.15 (t, J = 7.0 Hz, 3H, OCH_2CH_3), 1.00 (m, 1H, C-9 C*H*), 1.04 (d, J = 7.0 Hz, 3H, C-9 CH_3), 0.79-0.86 (m, 9H, C-8, C-11 and C-12 CH_3's);

^{13}C nmr ($CDCl_3$): 209.39 (C-5), 172.59 (C-1), 59.65 (OCH_2CH_3), 44.44 (C-6), 42.79 (C-4), 36.96 (C-9), 36.49 and 34.65 (C-2), 34.46 (C-3), 26.12 (C-10), 16.76 (C-7), 14.70 (C-12), 14.54 (OCH_2CH_3), 11.49 (C-8 and C-11);

hreims: m/z calculated for $C_{14}H_{26}O_3$: 242.1882; found 242.1875 (3.0), 199 (37), 197 (70), 157 (70), 111 (75), 83 (38), 71 (100).

Anal. Calcd. for $C_{14}H_{26}O_3$: C, 69.38; H, 10.81. Found C, 69.02; H, 10.73.

2-ethyl-5-(1-methylpropyl)-1,3-cyclohexanedione (52): from 92

Absolute ethanol (20 mL) was added to a suspension of sodium hydride (36 mg of a 60% suspension in mineral oil; 0.94 mmol) in toluene (20 mL) under an argon atmosphere. After evolution of hydrogen had ceased, a solution of keto ester 92 (228 mg, 0.94 mmol) in absolute ethanol (5 mL) was added. The solution was allowed to reflux for 24 hours. The cooled solution was extracted with dil. NaOH (3 x 50 mL of a 1*N* solution). The combined aqueous extracts were poured into ice cold 1*N* HCl (250 mL), at which point a precipitate formed. The mixture was extracted with dichloromethane (3 x 200 mL). The combined organic extracts were washed with brine (100 mL), dried ($MgSO_4$), and evaporated to yield crude dione 52. Purification by flash chromatography afforded pure dione (123 mg, 0.63

mmol; 67%).

2-ethyl-2-methyl-5-(1-methylpropyl)-1,3-cyclohexanedione (43): from 52

Clean potassium metal (1.33 g, 34 mmol) was added to fresly distilled *tert*-butyl alcohol (100 mL). After the potassium was completely dissolved, a solution of dione 52 (6.1 g, 31 mmol) in *tert*-butyl alcohol (25 mL) was slowly added. Iodomethane (4.2 mL, 68 mmol) was added. The mixture was allowed to reflux gently for 30 hours. The cooled reaction mixture was diluted with water (100 mL) and extracted with diethyl ether (4 x 100 mL). The combined ether extracts were washed with brine (2 x 100 mL), dried ($MgSO_4$), and evaporated to yield a yellow oil. Separation by flash chromatography afforded pure dione 43 as a colorless oil (4.15 g, 19.7 mmol; 64%) and keto ester 96 (1.80 g, 7.4 mmol; 24%).

Dione 43:

R_f 0.38 (dichloromethane);
ftir (chloroform cast): 2960, 1721, 1694 (s) and 1458 cm^{-1};
^1H nmr ($CDCl_3$): 2.3 - 2.5 (m, 4H, C-4 and C-6 CH_2's), 1.9 (m, 1H, C-5 C*H*), 1.81 (q, J = 7.0 Hz, 2H, C-11 CH_2), 1.4 (m, 2H, C-8 CH_2), 1.2 (m, 1H, C-7 C*H*), 1.18 (major) and

1.15 (minor) (s, 3H, C-13 CH_3), 0.9 (m, 6H, C-9 and C-10 CH_3's), 0.74 (t, J = 7.0 Hz, 3H, C-12 CH_3);

hreims : m/z calcd. for $C_{13}H_{22}O_2$: 210.1620; found 210.1621 (37), 111 (100).

Anal. Calcd. for $C_{13}H_{22}O_2$: C, 74.24; H, 10.54. Found C, 74.03; H, 10.44.

Methyl 3-(1-methylpropyl)-6-methyl-5-oxooctanoate (96):

R_f 0.20 (dichloromethane);

ftir (chloroform cast): 2962, 1733, 1712, 1461, 1438, 1379 and 1163 cm^{-1};

^1H **nmr** ($CDCl_3$): 3.65 (s, 3H, $COOCH_3$), 2.4 (m, 6H, C-3 and C-6 CH's, C-2 and C-4 CH_2's), 1.62 (oct, J = 7.0 Hz, 1H, C-7 CHH), 1.1-1.4 (m, 4H, C-11 CH_2 and C-7 CHH), 1.07 (d, J = 7.0 Hz, 3H, C-9 CH_3), 0.88 (m, 9H, C-8, C-12 and C-13 CH_3's);

^{13}C **nmr** ($CDCl_3$): 210.17 (C-5), 173.63 and 173.34 (C-1), 51.31 (OCH_3), 47.78 (C-6), 43.07 and 41.35 (C-4), 37.33 (C-9), 36.69 and 34.78 (C-2), 34.50 and 34.86 (C-3), 26.61 and 26.38 (C-10), 25.54 and 25.47 (C-7), 15.74 and 15.09 (C-13), 14.87 (C-12), 11.88 and 11.49 (C-8 and C-11);

hreims : m/z calculated for $C_{14}H_{26}O_3$: 242.1882; found 242.1879 (4.0), 185 (100), 171 (19), 143 (63), 111 (71), 83 (51), 71 (51).

Anal. Calcd. for $C_{14}H_{26}O_3$: C, 69.38; H, 10.81. Found C, 69.24; H, 10.53.

1,3-bis(trimethylsilyloxy)-2-ethyl-2-methyl-5-(1-methylpropyl)-3,6-cyclohexadiene (105)

Sodium iodide (750 mg, 5 mmol) dissolved in acetonitrile (5 mL) was added to a solution of dione 43 (166 mg, 0.79 mmol), triethylamine (0.69 mL, 5 mmol) and chlorotrimethylsilane (0.63 mL, 5 mmol). The resulting suspension was stirred at r.t. for 18 hours. The solvent was removed under reduced pressure. The resulting brown residue was triturated with dry cyclohexane (4 x 10 mL), filtered, and the cyclohexane evaporated under reduced pressure to give 105 as a pale yellow oil (261 mg, 0.74 mmol; 93%).

R_f 0.94 (dichloromethane);

ftir (chloroform cast): 2961, 1687, 1650, 1459, 1320, 1262, 1252, 1150, 1127, 917 and 844 cm^{-1};

^1H nmr ($CDCl_3$): 4.62 (bd, J = 3.0, 2H, C-4 and C-6 CH), 2.89 (m, 1H, C-5 CH), 1.48 (m, 2H, C-11 CH_2), 1.35 (m, 2H, C-8 CH_2), 1.1 (m, 1H, C-7 CH), 1.13 and 1.09 (s, 3H, C-13 CH_3), 0.90 (t, J = 7.0 Hz, 3H, C-9 CH_3), 0.81 and 0.78 (d, J = 7.0 Hz, 3H, C-10 CH_3), 0.68 (t, J = 7.0 Hz, 3H, C-12 CH_3), 0.18 (s, 18H, $Si(CH_3)_3$);

cims : m/z 355 (M+1, 100);

hreims : m/z calculated for $C_{18}H_{35}O_2Si_2$ ($M-CH_3$): 339.2175; found 339.2178 (2), 325 (2), 297 (100), 73 (68).

2-ethyl-2-methyl-5-(1-methylpropyl)-4-cyclohexene-1,3-dione

(41)

Dichlorodicyanobenzoquinone (39 mg, 0.17 mmol) dissolved in benzene (2 mL), was added to a solution of silyl enol ether 105 (61 mg, 0.17 mmol) in benzene (2 mL). The orange color of the solution is allowed to disappear then the solution is stirred for a further 5 min. The solution was filtered over a 1 cm thick bed of alumina and the filtrate evaporated under reduced pressure. The residue was dissolved in dichloromethane. Separation by flash chromatography gave enone 41 (7.4 mg, 0.036 mmol; 21%), dione 43 (11.7 mg, 0.056 mmol; 33%), and silyl enol ether 106 (6.2 mg, 0.022 mmol; 13%).

Enone 41:

R_f 0.28 (dichloromethane);
ftir (chloroform cast): 2965, 2933, 1726, 1665, 1460, 1382 and 1119 cm^{-1};
^1H nmr ($CDCl_3$): 6.08 (br s, 1H, C-4 C*H*), 3.20 (m, 2H, C-6 CH_2), 2.31 (m, 1H, C-10 C*H*), 1.79 and 1.82 (q, J = 7.0 Hz, 2H, C-7 CH_2), 1.5 (m, 1H, C-11 CH_2), 1.25 (s, 3H, C-9 CH_3), 1.13 (d, J = 7.0 Hz, 3H, C-13 CH_3), 0.89 and 0.87 (t, J = 7.0 Hz, 3H, C-12 CH_3), 0.83 (t, J = 7.0 Hz, 3H, C-8 CH_3);
hreims : m/z calculated for $C_{13}H_{20}O_2$: 208.1463; found 208.1465 (42), 190 (8), 180 (7), 175 (33), 96 (100), 84 (32).

2-Ethyl-2-methyl-5-(1-methylpropyl)-3-trimethylsilyloxy-3-cyclohexenone (106):

R_f 0.58 (dichloromethane);
ftir (chloroform cast): 2963, 2933, 2876, 1715, 1655, 1457, 1379, 1370, 1346, 1264, 1253, 1214, 1191, 1168, 1141, 925, 912, 882, 848 and 752 cm^{-1};
^1H nmr ($CDCl_3$): 4.90 (m, 1H, C-6 CH), 3.4 (m, 3H, C-4 CH_2 and C-5 CH), 1.2-1.9 (m, 5H, C-8 and C-11 CH_2's, C-10 CH), 1.16 (s, 3H, C-7 CH_3), 0.90 (m, 6H, C-10 and C-13 CH_3's), 0.72 (m, 3H, C-9 CH_3), 0.24 (s, 9H, $Si(CH_3)_3$);
hreims : m/z calculated for $C_{16}H_{30}O_2Si$: 282.2015; found 282.2018 (6.5), 239 (24), 225 (100).
Anal. Calcd. for $C_{16}H_{30}O_2Si$: C, 68.02; H, 10.70. Found C, 67.71; H, 10.37.

Attempted formylation of 41

a) Enone 41 (3.0 mg, 0.014 mmol) in dry THF (1 mL) was added dropwise to a stirred suspension of sodium hydride (5.5 mg of a 60% suspension, 0.14 mmol) in dry THF (2 mL), under an argon atmosphere. The solution immediately turns bright yellow. After stirring for 15 min, ethyl formate (11 uL, mmol) was added. The solution was allowed to stir at r.t. for 7 days at which time no trace of formylated product could be detected by tlc ($FeCl_3$ visualization). The solution was poured into cold dil HCl

and the resulting mixture extracted with Et_2O (3 x 50 mL). The combined Et_2O layers were washed with brine, dried ($MgSO_4$), and evaporated to yield a yellow oil. The ^1H nmr spectrum of the crude oil displays no signal characteristic of a formyl proton and corresponds to unreacted enone 41.

b) Enone 41 (2.7 mg, 0.013 mmol) in dry ethyl formate (0.5 mL) was added to a suspension of sodium hydride (7 mg of a 60% suspension, 0.18 mmol) in dry ethyl formate (0.5 mL) inside a pressure tube (0.5 cm internal diameter). The tube was sealed and immersed in a steam bath for 2 days. After cooling, tlc analysis ($FeCl_3$) showed no trace of formylated product. Work up as in a) and ^1H nmr analysis of the crude reaction product showed mainly unreacted starting material.

2-ethyl-2-methyl-4-hydroxymethylene-5-(1-methylpropyl)-1,3-cyclohexanedione (42)

n-Butyl lithium (2.00 mL of 1.57*N* solution in hexanes, 3.15 mmol) was added to a stirred solution of diisopropylamine (0.53 mL, 3.75 mmol) in dry THF (10 mL) at -78° under argon. After stirring for 15 min, a solution of dione 2 (315 mg, 1.50 mmol) in dry THF (5 mL) was added dropwise. After further stirring for 90 min, ethyl formate (121 uL, 1.50 mmol) was added. The cooling bath was removed and the reaction allowed to warm to r.t.. After stirring for 40

hours, the suspension was poured into dil. sodium hydroxyde (20 mL of 0.5*N* aqueous sol.). The aqueous layer was extracted with diethyl ether (3 x 50 mL), then acidified with 1*N* HCl, and extracted with dichloromethane (3 x 50 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and evaporated to yield crude α-formyl ketone 42 (286 mg). Separation by flash chromatography afforded pure 42 (218 mg, 0.915 mmol; 61% yield), and bisformylated product 121 (75 mg, 0.28 mmol; 19%).

Formylketone 42:

R_f 0.53 (dichloromethane);
ftir (chloroform cast): 2968, 2936, 1717, 1633, 1590, 1460 cm^{-1};
^1H nmr ($CDCl_3$): 15.07 (bm, 1H, CHO*H*), 8.71 (bm, 1H, C*H*OH), 2.6 (m, 3H, C-5 C*H* and C-6 CH_2), 1.9 (m, 2H, C-7 CH_2), 1.31 and 1.29 (s, 3H, C-9 CH_3), 0.7 - 1.1 (m, 12H, C-11 C*H*, C-12 CH_2, C-8, C-13 and C-14 CH_3's);
hreims : m/z calculated for $C_{14}H_{22}O_3$: 238.1569; found 238.1570 (4.7), 181 (100), 163 (22), 97 (70), 85 (46), 57 (27).

2-Ethyl-2-methyl-4-6-bishydroxymethylene-5-(1-methylpropyl)-1,3-cyclohexanedione (112):

R_f 0.58 (dichloromethane);

ftir (chloroform cast): 2967, 2936, 2877, 1631, 1606, 1461, 1374, 1343, 1312, 1300, 1206, 1184, and 1161 cm^{-1};

^1H nmr ($CDCl_3$): 15.02 (t, J = 3.0 Hz, 2H, CHO*H*'s), 8.86 and 8.76 (d, 1H, J = 3.0 Hz, C*H*OH's), 3.34 (d, J = 3.0 Hz, 1H, C-5 C*H*), 1.97 (q, J = 7.0 Hz, 2H, C-7 CH_2), 1.45 (s, 3H, C-9 CH_3), 1.3 (m, 1H, C-10 C*H*), 0.83 (d, J = 7.0 Hz, 3H, C-13 CH_3), 0.7 - 0.8 (m, 5H, C-11 CH_2 and C-12 CH_3), 0.59 (t, J = 7.0 Hz, 3H, C-9 CH_2);

hreims : m/z calculated for $C_{15}H_{22}O_4$: 266.1518; found 266.1510 (0.5), 209 (100), 181 (27), 153 (16), 125 (13).

4-acetoxymethylene-2-ethyl-2-methyl-5-(1-methylpropyl)-1,3-cyclohexanedione (120)

Pyridine (2 mL) was added to a solution of α-formyl ketone (218 mg, 0.915 mmol) in acetic anhydride (2 mL). The reaction mixture was stirred at r.t. overnight (*ca* 10 hours). The solvents were removed under reduced pressure (Buchi evaporator) and the residue dissolved in dichloromethane. Purification by flash chromatography afforded pure acetate 120 as an oil (247 mg, 0.983 mmol; 96%).

R_f 0.28 (dichloromethane);

ftir (chloroform cast): 2960, 1777, 1685, 1368, 1193 and 1169 cm^{-1};

^1H nmr ($CDCl_3$): 8.01 and 8.06 (br s, 1H, CHOAc), 3.04 (m, 1H, C-5 CH), 2.75 (m, 2H, C-6 CH_2), 2.27 (major) and 2.32 (minor) (s, 3H, $OCOCH_3$), 1.9 (m, 2H, C-7 CH_2), 1.26 (minor) and 1.22 (major) (s, 3H, C-9 CH_3), 1.0-1.5 (bm, 3H, C-11 CH and C-12 CH_2), 0.7 - 0.9 (m, 9H, C-8, C-13 and C-14 CH_3's); hreims : m/z calculated for $C_{16}H_{24}O_4$: 280.1674; found 280.1673 (25), 238 (16), 181 (100), 125 (28), 97 (21).

2-ethyl-2-methyl-4-hydroxymethylene-5-(1-methylpropyl)-1-trimethysilyloxy-6-cyclohexen-3-one (119)

Sodium iodide (662 mg, 4.4 mmol) in dry acetonitrile (2 mL) was added dropwise under argon to a solution of acetoxy ketone 120 (247 mg, 0.883 mmol), triethylamine (0.61 mL, 4.4 mmol) and chlorotrimethylsilane (0.56 mL, 4.4 mmol). The resulting suspension was stirred at r.t. for 20 hours. The solvent was removed under reduced pressure. The resulting brown residue was triturated with dry cyclohexane, filtered, and the cyclohexane evaporated under reduced pressure to give crude 119. Separation by flash chromatography afforded pure silyl enol ether 119 as a oil (216 mg, 0.697 mmol; 79%) and acetyl silyl enol ether 121

Silyl enol ether 119:

R_f 0.70 (dichloromethane);

ftir (chloroform cast): 2925, 1670, 1451, 1373, 1245, 1148 and 840 cm^{-1};

^1H nmr ($CDCl_3$): 14.9 (m, 1H, CHOH), 9.05 and 9.01 (d, J = 8.0 Hz, 1H, CHOH), 4.80 (m, 1H, C-6 CH), 3.31 (m, 1H, C-5 CH), 1.76 (q, 2H, C-7 CH_2), 1.30 (minor) and 1.25 (major) (s, 3H, C-13 CH_3), 1.1-1.6 (bm, 3H, C-11 CH and C-12 CH_2), 1.00 (t, J = 7.0 Hz, 3H, C-8 CH_3), 0.6 - 0.9 (m, 6H, C-13 and C-14 CH_3's), 0.28 (s, 9H, $Si(CH_3)_3$);

cims : 328 (M+18, 100%);

hreims : m/z calculated for $C_{13}H_{21}O_3Si$ ($M-C_4H_9$): 253.1260; found 253.1257 (96), 225 (55), 197 (10), 73 (51), 57 (100).

2-Ethyl-2-methyl-4-acetoxymethylene-5-(1-methylpropyl)-1-trimethysilyloxy-6-cyclohexen-3-one (121):

R_f 0.27 (dichloromethane);

ftir (chloroform cast): 2964, 2935, 1777, 1689, 1671, 1622, 1459, 1372, 1264, 1253, 1228, 1197, 1176, 1167 and 847 cm^{-1};

^1H nmr ($CDCl_3$): 7.9 (m, 1H, CHOAc), 4.85 (m, 1H, C-6 CH), 3.45 (m, 1H, C-5 CH), 2.19 (s, 3H, $COCH_3$), 1.7-1.1 (m, 5H, C-7 and C-11 CH_2's and C-10 CH), 1.10 (minor) and 1.18 (major) (s, 3H, C-9 CH_3), 0.92 and 0.83 (m, 2 x 3H, C-12 and C-13 CH_3's), 0.65 (m, 3H, C-8 CH_3), 0.20 and 0.21 (major) (s, 9H, $Si(CH_3)_3$);

cims : 370 (M+18, 100%);

hreims : m/z calculated for $C_{19}H_{32}O_4Si$: 352.2070; found 352.2070 (i), 295 (32), 253 (100), 225 (39), 209 (32), 73 (63), 57 (38).

2-ethyl-2-methyl-4-hydroxymethylene-5-(1-methylpropyl)-5-cyclohexene-1,3-dione (1)

Dichlorodicyanobenzoquinone (35 mg, 0.15 mmol) dissolved in benzene - chloroform (1:1) (5 mL), was added to a solution of silyl enol ether 119 (48 mg, 0.15 mmol) in benzene (5 mL). The solution was allowed to stir for 6 hours. The precipitate formed was removed by filtration over celite and the filtrate evaporated under reduced pressure. The residue was dissolved in dichloromethane - methanol (97:3). Separation by flash chromatography gave ceratenolone 1 (10.2 mg, 0.043 mmol; 28%) and adduct 122 (29.8 mg, 0.065 mmol; 43%).

Ceratenolone (1):

R_f 0.21 (dichloromethane), 0.69 (chloroform - acetone - acetic acid, 23:1:1);

ftir (chloroform cast): 2967, 2933, 1660, 1613 (s), 1574, 1454, 1303, 1277 and 1258 cm^{-1};

^1H nmr ($CDCl_3$): 16.31 (d, J = 8.0 Hz, 1H, CHO*H*), 8.71 (d, J = 8.0 Hz, 1H, C*H*OH), 5.84 (s, 1H, C-6 C*H*), 2.55 (sext, 1H, C-10 C*H*), 1.89 (q, J = 7.0 Hz, 2H, C-7 CH_2), 1.6 (m, 1H,

C-11 CH_2), 1.29 (s, 3H, C-9 CH_3), 1.14 (d, J = 7.0 Hz, 3H, C-13 CH_3), 0.89 (t, J = 7.0 Hz, 3H, C-12 CH_3), 0.69 (t, J = 7.0 Hz, 3H, C-8 CH_3);

hreims : m/z calculated for $C_{14}H_{20}O_3$: 236.1412; found 236.1410 (75), 208 (96), 193 (56), 179 (100), 175 (31), 151 (34), 123 (33), 57 (35), 55 (51).

2-Ethyl-2-methyl-4-hydroxymethylene-5-(1-methylpropyl)-6-(2,3-dichloro-5,6-dicyano-4-hydroxyphenoxy)-5-cyclohexene-1,3-dione (122):

R_f 0.34 (chloroform - acetone - acetic acid, 23:1:1);

ftir (methanol cast): 3405 (br), 2962, 2935, 2855, 2230, 1628, 1608, 1450, 1436, 1409, 1383, 1353, 1299, 1253, 1212, 1188, 1085, 1025, 915 cm^{-1};

^1H nmr (acetone-d_6): 9.05 and 9.09 (2 x s, 0.2H), 8.05 (br s), 7.55 (br s), 3.55 (m), 1.5-1.9 (4H), 1.0-1.3 (6H), 0.6-1.0 (6H);

hreims: m/z calculated for $C_{22}H_{20}N_2O_5Cl_2$: 462.0749; found 462.0740 (0.6), 444 (6), 85 (56), 57 (100).

3-7-Dimethyl-5-nonen-4-one (45)

n-Butyllithium (28.8 mL of a 1.55*N* solution in hexanes, 45 mmol) was added to a solution of diisopropylamine (9.4 mL,

67 mmol) in dry THF (100 mL) under argon at -78°. After stirring for 30 min, 2-pentanone (4.7 mL, 45 mmol) was added dropwise. This solution was further stirred for 60 min and 2-methylbutyraldehyde (5.0 mL, 47 mmol) was rapidly added. After further stirring for 10 min at -78°, sat. NH_4Cl (5 mL) was added. The solution was allowed to warm to room temperature, then poured into a mixture of cold 10% HCl (200 mL) and diethyl ether (100 mL). The organic layer was separated, washed with water, brine, dried ($MgSO_4$), and the solvent evaporated under reduced pressure to give the crude β-hydroxyketone.

The crude β-hydroxyketone was dissolved in benzene (100 mL) and *p*-toluenesulphonic acid (1 g) was added. After stirring for 15 min, 4A molecular sieves (30 g) were added and the mixture was allowed to stand at room temperature for 24 hours. The resulting brown solution was filtered, washed with 5% $NaHCO_3$, water, brine, dried ($MgSO_4$), and the solvent evaporated to give the crude enone (6.5 g). Purification by flash chromatography afforded pure enone 45 (5.9 g, · mmol; 78%) ;

R_f 0.48 (dichloromethane);

ftir (chloroform cast): 2966, 2934, 2877, 1668, 1623, 1460, 1379, 1200 and 982 cm^{-1};

^1H nmr ($CDCl_3$): 6.73 (dd, J = 16.0, 8.0 Hz, 1H, C-6 *H*), 6.09 (dd, J = 16.0, 1.5 Hz, 1H, C-5 *H*), 2.66 (sext, J = 7.0 Hz, 1H, C-3 *H*), 2.20 (br sept, J = 7.0 Hz, 1H, C-7 *H*), 1.65 (dquint, J = 14.0, 7.0 Hz, 1H, C-2 *H*), 1.38 (quint, J = 7.0

Hz, 2H, C-8 CH_2), 1.36 (dquint, J = 14.0, 7.0 Hz, 1H, C-2 H), 1.04 (d, J = 7.0 Hz, 3H, C-10 CH_3), 1.00 (d, J = 7.0 Hz, 3H, C-11 CH_3), 0.84 (t, J = 7.0 Hz, 6H, C-1 and C-9 CH_3's);

^{13}C nmr ($CDCl_3$): 204.00 (C-4), 152.29 (C-6), 127.27 (C-5), 45.26 (C-3), 38.25 (C-7), 28.78 (C-8), 26.14 (C-2), 18.94 (C-10), 16.05 and 15.80 (C-11), 11.55 and 11.47 (C-1 and C-9);

hreims : m/z calculated for $C_{11}H_{20}O$: 168.1514; found 168.1512 (9), 111 (64), 85 (36), 83 (100), 57 (71), 55 (30).

3-(1-methylpropyl)-6-methyl-2-carboethoxy-5-oxooctanoic acid (133)

Enone (1.30 g, 7.7 mmol) was added to a solution of magnesium monoethyl malonate (2.4 g, 15 mmol) in freshly distilled dimethylformamide (20 mL) . The solution was warmed to 60° and stirred for 26 hours. The cooled solution was poured into a mixture of cold 5% aqueous KOH (100 mL) and ether (100 mL). The aqueous layer was separated and acidified with cold 10% HCl, then extracted with ether (3 x 80 mL). The combined ether extracts were washed with water, brine, dried ($MgSO_4$), and the solvent evaporated under reduced pressure to give the crude acid. Purification by flash chromatography provided an analytical sample of acid 133.

R_f 0.08 (dichloromethane);

ftir (chloroform cast): 3500-2400 (br), 2965, 1735, 1714, 1461, 1376, 1179 and 1158 cm^{-1};

^1H nmr ($CDCl_3$): 9.90 (br s, 1H, COO*H*), 4.2 (m, 2H, OCH_2CH_3), 3.68 (m, 1H, C-2 *H*), 2.5 (m, 4H, C-3 *H*, C-4 CH_2 and C-6 *H*), 1.78 (oct, J = 7.0 Hz, 1H, C-7 CH*H*), 1.4 (m, 3H, C-11 CH_2 and C-7 CH*H*), 1.25 (t, 3H, OCH_2CH_3), 1.1 (m, 1H, C-10 *H*), 1.06 and 1.05 (d, J = 7.0 Hz, 3H, C-9 CH_3), 0.86 (m, 9H, C-8, C-12 and C-13 CH_3's);

cims : 318 (100) (M + 18);

hreims : m/z calculated for $C_{16}H_{28}O_5$: 300.1936; found 300.1938 (1.1), 243 (58), 225 (54), 197 (56), 169 (37), 137 (50), 111 (43), 85 (49), 57 (100).

Ethyl 3-(1-methylpropyl)-6-methyl-5-oxooctanoate (98)

The crude acid was placed in a 5 mL flask and heated to 160° in an oil bath for 15 min. After cooling the oil to 25°, the crude ester was purified by flash chromatography to give ketoester 98 (0.41 g, 1.6 mmol; 20%) and unreacted enone (0.55 g, 3.3 mmol; 42%).

R_f 0.31 (dichloromethane);

ftir (chloroform cast): 2964, 2935, 1733, 1711, 1460, 1372, 1175 and 1159 cm^{-1};

^1H nmr ($CDCl_3$): 4.11 (q, J = 7.0 Hz, 2H, OCH_2CH_3), 2.5 (m, 6H, C-3 and C-6 *H*'s, C-2 and C-4 CH_2's), 1.68 (oct, J = 7.0 Hz, 1H, C-7 CH*H*), 1.4 (m, 3H, C-11 CH_2 and C-7 CH*H*), 1.24

(t, J = 7.0 Hz, 3H, OCH_2CH_3), 1.1 (m, 1H, C-10 *H*), 1.04 (d, J = 7.0 Hz, 3H, C-9 CH_3), 0.88 (m, 9H, C-8, C-12 and C-13 CH_3's);

^{13}C nmr ($CDCl_3$): 213.74 and 213.66 (C-5), 172.95 (C-1), 60.18 and 60.13 (OCH_2CH_3), 47.87 and 47.81 (C-6), 43.45 and 41.70 (C-4), 37.06 (C-9), 36.78 and 35.06 (C-2), 34.56 (C-3), 26.68 and 26.43 (C-10), 25.89 (C-7), 15.80 and 15,22 (C-13), 14.92 (C-12), 14.15 (OCH_2CH_3), 11.95 (C-8), 11.56 (C-11);

hreims : m/z calculated for $C_{15}H_{28}O_3$: 256.2038; found 256.2031 (3.9), 199 (83), 171 (18), 157 (42), 111 (59), 85 (34), 83 (38), 57 (100).

3-(1-Methylpropyl)-6-methyl-5-oxooctanoic acid (44)

Potassium hydroxide (10 mL of 1*N* sol.) was added to a solution of keto ester (410 mg, 1.6 mmol) in 95% ethanol (10 mL) . The solution was heated to reflux for 5 hours, cooled, and poured onto 5% HCl (100 mL). The solution was extracted with dichloromethane (2 x 100 mL). The organic extracts were combined, washed with water, brine, dried ($MgSO_4$), and the solvent evaporated under reduced pressure to give the crude acid. Purification by flash chromatography afforded pure acid (354 mg, 1.5 mmol; 97%);

R_f 0.23 (dichloromethane-methanol, 98:2);

ftir (chloroform cast): 3400-2400 (b), 2964, 2934, 1735, 1708, 1462 and 1381 cm^{-1};

^{1}H nmr ($CDCl_3$): 10.0 (br s, 1H, COOH), 2.5 (m, 6H, C-3 and C-6 H's, C-2 and C-4 CH_2's), 1.71 (oct, J = 7.0 Hz, 1H, C-7 CHH), 1.35 (m, 3H, C-11 CH_2 and C-7 CHH), 1.05 (m, 1H, C-10 H), 0.99 and 0.98 (d, J = 7.0 Hz, 3H, C-9 CH_3), 0.86 (m, 9H, C-8, C-12 and C-13 CH_3's);

^{13}C nmr ($CDCl_3$): 213.32 (C-5), 178.60 (C-1), 47.68 and 47.58 (C-6), 44.88 and 43.20 (C-4), 36.89 (C-9), 36.25 and 34.62 (C-2), 34.25 and 34.09 (C-3), 26.35 and 26.14 (C-10), 25.82 (C-7), 15.39 and 15,28 (C-13), 14.86 (C-12), 11.46 (C-11), 11.09 (C-8);

hreims : m/z calculated for $C_{13}H_{24}O_3$: 228.1725; found 228.1726 (3.5), 171 (86), 153 (18), 111 (25), 85 (37), 83 (49), 72 (62), 57 (100).

Anal. Calcd. for $C_{13}H_{24}O_3$: C, 68.38; H, 10.59. Found C, 68.46; H, 10.61.

Attempted formation of dione 43

Acetic anhydride (25 uL, 0.27 mmol) and boron trifluoride etherate (30 uL, 0.27 mmol) were added to a solution of ketoacid 44 (27.9 mg, 0.12 mmol) in acetic acid (5 mL). The solution was stirred at room temperature for 12 hours under argon, then heated to reflux for 24 hours. The cooled solution was poured into a mixture of water (50 mL) and ether (50 mL). The ether layer was separated, washed with water, brine, dried ($MgSO_4$), and the excess ether evaporated under reduced pressure to give recovered

ketoacid (21 mg).

2-ethyl-2-methyl-5-(1-methylpropyl)-1,3-cyclohexanedione 43: from 44

p-Toluenesulphonic acid (5 mg) was added to a solution of 44 (51.3 mg, 0.23 mmol) in toluene (20 mL). The solution was heated to reflux for 24 hours using a Dean Stark apparatus. The solution was cooled and extracted with 5% Na_2CO_3 (2 x 25 mL). The organic layer was separated, dried ($MgSO_4$), and evaporated under reduced pressure to yield a brown residue. Purification by flash chromatography (dichloromethane) afforded dione 43 (18 mg, 0.08 mmol; 38%).

D. Iron binding studies

Reagents

Ferric nitrate ($Fe(NO_3)_3.9(H_2O)$; Anachemia ,reagent grade) was used without crystallization. Water was doubly distilled from an all glass still. Stock solutions of Fe^{+3} were prepared by the dissolution of $Fe(NO_3).9(H_2O)$ into standardized nitric acid, followed by quantitative dilution

to give a final solution which was typically 10^{-4}M in iron and acid. Alternatively, $Fe(NO_3).9(H_2O)$ was dissolved in water immediatly before use in the titration experiment.

Potentiometric Measurements

All pH measurements were made with a Orion Research Model 701 digital pH meter equipped with Corning glass electrode. The apparatus was calibrated with standard buffer solutions (4.00 and 7.00) before each titrations. The pH of the solutions was adjusted to the desired value by the addition of concentratred nitric acid.

Spectrophotometric Measurements

Light absorption measurements were made with a Hewlett Packard 8450A diode array spectrophotometer. A square cuvette of optical thickness 1.00 cm was used throughout. After addition of titrant, an aliquot was removed and placed in the cuvette. Its visible spectrum was recorded and the aliquot returned to the titration vessel.

Titration apparatus

The titration vessel consists of a 30 mL beaker. A 10 mL aliquot of the solution to be titrated is placed in the beaker. The titrant is delivered from a graduated burette

(+/- 0.02 mL). The solution is stirred with a small magnetic stirring bar. A miniature electrode is used to measure the pH of the solution throughout the titration.

Titration of ceratenolone with ferric nitrate

A 10.0 mL solution of ceratenolone (6.7×10^{-4}M) in methanol-water (25:75) was placed in the titration vessel. The pH of the solution was adjusted to 2.61 by addition of *ca* 0.05 ml of concentrated HNO_3. An aliquot of this solution was used as the blank and was returned to the titration vessel. The solution was titrated with a ferric iron solution in water (4.46×10^{-3}M). Aliquots were taken after each drop of titrant was added (*ca* 0.06 ml), and the visible spectra and the absorbance at 550 nm recorded. A total volume of titrant of 2 mL was delivered.

Titration of ferric nitrate with ceratenolone

a) A 10.0 mL solution of ferric nitrate (5.12×10^{-5}M) in dil. HNO_3 (pH 2.61), was titrated as before, with a solution of ceratenolone in methanol-water (25:75) (1.22×10^{-3}M). A total volume of 1.6 mL was delivered.

b) A 10.0 mL solution of ferric nitrate (1.79×10^{-4}M) in dil. HNO_3 (pH 2.01), was titrated as before, with a

solution of ceratenolone in methanol-water (25:75) (1.22×10^{-3}M). A total volume of 8.5 mL was delivered.

Determination of the acidity constant of ceratenolone (1)

A solution of ceratenolone (10 mL of a 1.22×10^{-3}M sol.) was titrated with a standard solution of sodium hydroxide (2.1×10^{-3}M). The initial pH of the solution was 3.70. The endpoint was reached after 3.6 mL of titrant had been added. The pH of the solution at the half point was 4.41, thus $pK_a = 4.41$.

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